Remarks

Applicants request reconsideration on the merits of the above-referenced patent application.

I. Claim amendments

This amendment adds claims 12-20. Thus, claims 6-20 are pending. Claims 7, 8, and 11 have been amended. Applicants submit that the amendments and new claims do not introduce new matter. Specifically:

Claims 7 and 11 have been amended to replace abbreviations with the corresponding full names.

Claim 8 has been amended to be more specifically directed to a method where the vaccine is administered to a horse.

New claims 12 and 20 are directed to methods using vaccines of bacteria recited in Table 1 of the discussion below. These claims are supported by Applicants' specification at, for example, page 4, line 3 to page 5, line 29.

New claims 13-19 are directed to methods where the vaccine is administered to one of the four types of mammals originally recited in claim 8. These claims are supported by Applicants' specification at, for example, page 4, line 3 to page 5, line 25.

Applicants reserve the right to pursue any canceled subject matter and/or any other subject matter disclosed in this application in one or more divisional and/or continuation applications.

II. Acknowledgement of withdrawal of the written description rejection

The written description rejection set forth in the June 28, 2005 Office action has not been reiterated in the February 22, 2006 Office action. Thus, in accordance with the third paragraph on page 2 of the February 22, 2006 Office action, that rejection is deemed withdrawn.

III. Response to the double patenting rejections

Claims 6-11 have been rejected under the judicially-created doctrine of obviousness-type double patenting in view of claims 1-4 of U.S. Patent 6,682,745. Applicants submit that this rejection is premature because claims 6-11 have not yet been found to be otherwise allowable. Once the claims in this application have been found to be otherwise allowable, Applicants will further address the merits of this rejection and/or file a terminal disclaimer.

Claims 6-8 have been rejected under the judicially-created doctrine of obviousness-type double patenting as being unpatentable over claim 1 of U.S. Patent 6,120,775. Applicants submit that this rejection is premature because claims 6-8 have not yet been found to be otherwise allowable. Once the claims in this application have been found to be otherwise allowable, Applicants will further address the merits of this rejection and/or file a terminal disclaimer.

IV. Response to the enablement rejection

Claims 6-11 have been rejected under 35 U.S.C. 112 (first paragraph) for lacking enablement. Specifically, the Office action indicates that, while the specification is enabling as to methods using *S. equi*, it is not enabling as to methods using live vaccines generally. Applicants request withdrawal of this rejection.

Claims 6-8 are directed to a method for administering a live vaccine, and claims 9-11 are directed to a method for reducing the amount of adverse reactions at an injection site of a live bacteria vaccine. Applicants respectfully submit that this subject matter is enabled under well-established Federal Circuit law as set forth in, for example, the case cited in the Office action, *In re Wands*, 8 USPQ2d 1400 (Fed. Cir. 1988).

The claimed subject matter stems from Applicants' discovery that submucosal administration of live vaccines generally tends to reduce local reactions that had previously been observed when live vaccines were administered via conventional routes for systemic application (particularly intramuscular administration). This reduction in local reactions is advantageous because it, for example, generally allows for less-attenuated vaccines to be used. *See e.g.*, Applicants' specification, page 1, lines 20-24. Applicants' discovery is not limited to any single vaccine. Its general applicability to live vaccines is corroborated by

Applicants' working examples, which illustrate advantages of using submucosal administration with four different live bacterial strains and two different host species. *See* pages 7-9.

Armed with the teachings of Applicants' specification, along with the knowledge already in the art, a skilled artisan would have sufficient knowledge to administer any given live vaccine in the manner claimed. In general, a claim satisfies the enablement requirement if the specification enables a skilled artisan to make and use the claimed invention without "undue experimentation." The necessity for "complex" experimentation does not necessarily equate to "undue" experimentation if those in the art typically engage in such experimentation. See MPEP §2164.01. Claims are enabled even if "a considerable amount" of experimentation is necessary where the experimentation is "merely routine" or the specification "provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed." *Wands*, 8 USPQ2d at 1404. Whether a specification requires undue experimentation depends on multiple factors:

> (1) the quantity of experimentation necessary, (2) the amount of direction or guidance presented, (3) the presence or absence of working examples, (4) the nature of the invention, (5) the state of the prior art, (6) the relative skill of those in the art, (7) the predictability or unpredictability of the art, and (8) the breadth of the claims.

Wands, 8 USPQ2d at 1404. See *also*, MPEP §2164.01(a).

Applicants submit that their claims satisfy the enablement requirement for reasons analogous to those in *Wands*. More specifically, in *Wands*, the claims were directed to monoclonal IgM antibodies and an immunoassay using the antibodies. The court found that the claims were enabled even though a skilled artisan practicing the claimed invention would have to obtain lymphocytes from an immunized animal; fuse the lymphocytes with myeloma cells; and then perform multiple screening steps to identify and separate out hybridomas, hybridomas producing antibodies to the desired antigen, and finally hybridomas producing antibodies having the claimed affinity. *Wands*, 8 USPQ2d at 1404-1406. In finding enablement, the court noted: (1) the specification provided guidance for practicing the invention, (2) the specification provided working examples, (3) the level of skill in the art was high, (4) the methods needed to practice the invention were well known in the art, and (5) the

nature of the technology involved screening to identify antibodies with the desired characteristics. *Wands*, 8 USPQ2d at 1406.

Using analogous reasoning, Applicants' claims 6-11 also should be found to satisfy the enablement requirement. Specifically:

1. Applicants' specification provides considerable direction and guidance for practicing the invention. For example, Applicants' specification provides generally suitable sites for submucosal administration (*see, e.g.*, page 2, line 30 to page 3, line 2 and page 3, lines 18-23); administration depths and techniques (*see, e.g.*, page 3, lines 3-17); dosage ranges (*see, e.g.*, page 6, lines 1-8); suitable carrier materials (*see, e.g.*, page 6, lines 9-17); and suitable adjuvants (*see, e.g.*, page 6, lines 18-26). Applicants' specification also, for example, identifies a wide range of live bacteria that are generally suitable for use with their invention. *See, e.g.*, page 3, line 25 to page 5, line 29.

2. Applicants' specification provides three working examples illustrating submucosal administration with four different live vaccines and two different host species. *See* pages 7-9.

3. The skill level in the art and nature of the technology are analogous to those in *Wands*.

4. The methods needed to practice the invention are well known in the art. As to live vaccines in particular, there was an extensive understanding in the art relating to methods for making and generally using live vaccines at the time Applicants filed their application. The scientific literature from that time is replete with discussions relating to the development and use of live vaccines. This literature includes much information relating to the representative vaccines that Applicants' specification lists on pages 4 and 5 (and in claims 7 and 11). To evidence this, **Table I** below provides a list of references discussing live vaccines that the Undersigned located during a non-exhaustive literature search. These references, which are enclosed for the Examiner's convenience, specifically describe the preparation and use of live vaccines for various bacteria listed by Applicants.

Table I

Examples of Live Vaccines Known at the Time of Applicants' Filing

Live vaccine disclosed	Reference citation	Filing date (if applicable)	Publication date
Actinobacillus pleuropneumoniae	Inzana, T.J., U.S. Patent 5,429,818, entitled "Non-capsulated mutants of *Actinobacillus pleuropneumoniae* useful as vaccines"	Priority: December 6, 1991 Filed: June 24, 1993	July 4, 1995
Actinobacillus pleuropneumoniae	Segers, R.P.A.M., et al., U.S. Patent 6,013,266, entitled "Live attenuated bacteria of the species *Actinobacillus pleuropneumoniae*"	Priority: April 10, 1997 Filed: April 9, 1998	January 11, 2000
Actinobacillus pleuropneumoniae	Fuller, T.E., et al., U.S. Patent 5,925,354, entitled "Riboflavin mutants as vaccines against *Actinobacillus pleuropneumoniae*"	Priority: November 30, 1995 Filed: October 28, 1996	July 20, 1999
Bordetella bronchiseptica	Switzer, W.P., et al, U.S. Patent 4,225,583, entitled "Intra-respiratory vaccine for prevention of *Bordetella bronchiseptica* infection and method of use"	December 7, 1978	September 30, 1980
Brucella abortus	Adams, L.G., U.S. Patent 5,718,903, entitled "Vaccine comprising *Brucella abortus* which has O polysaccharide antigen absent"	Priority: March 30, 1987 Filed: February 14, 1994	February 17, 1998
Brucella abortus	McEwen, A.D., et al., "Bovine contagious abortion. The use of guinea-pigs in immunisation studies," *The Journal of Comparative Pathology and Therapeutics*, XLIX(2), 97-117 (June 30, 1036)	N/A	June 30, 1936
Clostridium perfringens	Segers, R.P.A.M., et al., U.S. Patent 6,610,300, entitled "*Clostridium perfringens* vaccine"	Priority: June 20, 1997 Filed: June 19, 1998	August 26, 2003
Corynebacterium pseudotuberculosis	Simmons, C.P., "Attenuation and vaccine potential of *aroQ* mutants of *Corynebacterium pseudotuberculosis*," *Infection and Immunity*, 65(8), pp. 3048-3056 (August, 1997)	N/A	August of 1997

Live vaccine disclosed	Reference citation	Filing date (if applicable)	Publication date
Corynebacterium pseudotuberculosis	Powell, R.J., et al., U.S. Patent 5,997,881, entitled "Method of making non-pyrogenic lipopolysaccharide or A"	Priority: November 22, 1995 Filed: February 19, 1997	December 7, 1999
Erysipelothrix rhusiopathiae	Sakano, T., et al., "Effect of attenuated *Erysipelothrix rhusiopathiae* vaccine in pigs infected with porcine reproductive respiratory syndrome virus," *Journal of Veterinary Medical Science*, 59(11), pp. 977-981 (November, 1997)	N/A	November of 1997
Erysipelothrix rhusiopathiae	Sawada, T., et al., "Cross protection of mice and swine inoculated with culture filtrate of attenuated *Erysipelothrix rhusiopathiae* and challenge exposed to strains of various serovars," *American Journal of Veterinary Research*, 48(2), pp. 239-242 (February, 1987)	N/A	February of 1987
Escherichia coli	Wilson, M.R., et al., "The influence of preparturient intramammary vaccination on immunoglobulin levels in bovine mammary secretions," *Immunology*, 23 , pp. 313-320 (1972)	N/A	1972
Mycobacterium bovis	Flesselles, B., et al., U.S. Patent 6,136,324, entitled "Attenuated strains of mycobacteria"	August 21, 1997	October 24, 2000
Mycobacterium bovis	Barry, III, C.E., et al., U.S. Patent 6,403,100, entitled "Method of attenuating pathogenic mycobacteria and strains of mycobacteria so attenuated"	Priority: July 10, 1997 Filed: July 9, 1998	Appl. published: January 21, 1999 Issued: June 11, 2002
Mycoplasma hyopneumoniae	Lloyd, L.C., et al., "Protection against enzootic pneumonia of pigs: intraperitoneal inoculation with live LKR strain of *Mycoplasma hyopneumoniae,"* Australian Veterinary Journal, 66(1), pp. 9-12 (January, 1989)	N/A	January of 1989

Live vaccine disclosed	**Reference citation**	**Filing date (if applicable)**	**Publication date**
Pasteurella haemolytica	Kucera, C.J., U.S. Patent 4,506,017, entitled "Modified *Pasteurella haemolytica* bacteria"	Priority: April 17, 1981 Filed: January 19, 1983	March 19, 1985
Pasteurella multocida	Maheswaran, S.K., U.S. Patent 3,855,408, entitled "Poultry vaccine"	July 16, 1973	December 17, 1974
Pasteurella multocida	Glisson, J.R., et al., U.S. Patent 4,999,191, entitled "*Pasteurella multocida* Vaccine"	May 5, 1988	March 12, 1991
Rhodococcus equi	Chirino-Trejo, J.M., et al., "Protection of foals against experimental *Rhodococcus equi* pneumonia by oral immunization," *Canadian Journal of Veterinary Research*, 51, pp. 444-447 (1987)	N/A	1987
Salmonella choleraesuis	Smith, H.W., U.S. Patent 3,364,117, entitled "Vaccine for combating *Salmonella choleraesuis* infection"	Priority: September 10, 1963 Filed: September 9, 1964	January 16, 1968
Salmonella dublin	Stocker, B.A.D., U.S. Patent 4,550,081, entitled "Non-reverting salmonella"	Priority: May 19, 1980 Filed: September 7, 1982	October 29, 1985
Salmonella typhimurium	Stocker, B.A.D., U.S. Patent 4,550,081, entitled "Non-reverting salmonella"	Priority: May 19, 1980 Filed: September 7, 1982	October 29, 1985
Staphylococcus aureus	Australian Patent Appl. AU198285929A1, entitled "Mastitis vaccine"	July 12, 1982	January 20, 1983
Streptococcus pneumoniae	Helms, C.M., "Temperature-sensitive mutants of type I *Streptococcus pneumoniae*: preparation, characterization, and evidence for attenuation and immunogenicity," *The Journal of Infectious Diseases*, 136 (Supp.), pp. S208-S215 (August, 1977)	N/A	August of 1977

Live vaccine disclosed	Reference citation	Filing date (if applicable)	Publication date
Streptococcus suis	Quessy, S., et al., "Immunization of mice against *Streptococcus suis* serotype 2 infections using a live avirulent strain," *Canadian Journal of Veterinary Research (Short Communications)*, 58, pp. 299-301 (1994)	N/A	1994
Streptococcus suis	Busque, P., et al., "Immunization of pigs against *Streptococcus suis* serotype 2 infection using a live avirulent strain," *Canadian Journal of Veterinary Research*, 61, pp. 275-279 (1997)	N/A	1997
Streptococcus uberis	Hill, A.W., et al., "Immune modification of the pathogenesis of *Streptococcus uberis* mastitis in the dairy cow," *FEMS Immunology and Medical Microbiology*, 9, pp. 109-118 (1994)	N/A	1994
Streptococcus uberis	Finch, J.M, et al., "Further studies on the efficacy of a live vaccine against mastitis caused by *Streptococcus uberis*," *Vaccine*, 15(10), pp. 1138-1143 (1997)	N/A	1997

To the extent these references constitute knowledge in the art at the time of Applicants' filing, Applicants were not required to include any teachings from them in the specification. Nor were Applicants required to include any other teachings that were already known in the art at the time of Applicants' filing. The Patent Office, in fact, discourages inclusion of such material. *See* MPEP 2164.01 (a patent "preferably omits" anything that is already well known in the art).

A finding of enablement also is supported by the Federal Circuit's recent holding in *Falkner v. Inglis*, Case No. 05-1324 (Fed. Cir., May 26, 2006) (appeal from Interference No. 105,187). In that case, the court found that the Board did not err in finding enablement for a poxvirus vaccine claim, even in the absence of a poxvirus working example, where there was a working example for a herpesvirus vaccine. *Id.* at 12. In justifying its holding, the court

acknowledged that "great expenditures of time and effort were ordinary in the field of vaccine preparation." *Id.*

Simply put, the law does not require Applicants' specification to specifically describe every possible embodiment falling within the claims. *See In re Angstadt*, 190 USPQ 214, 218 (CCPA 1976) (patent applicants are not required to disclose every species encompassed by their claims, even in an unpredictable art). Such a requirement would be unfairly burdensome and prohibitive for Applicant, and would ultimately result in Applicants' claims being arbitrarily limited to less than the entire scope of their invention. *Id.* In view of the foregoing, Applicants submit that the enablement rejection of claims 6-11 must be withdrawn.

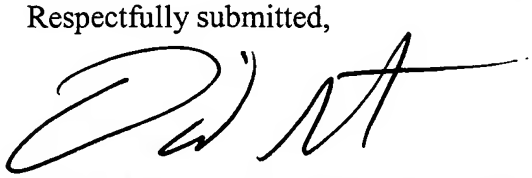
New claims 12-20 depend directly or indirectly from claim 6 or 9, and therefore necessarily satisfy the enablement requirement for at least the same reasons as discussed above for claims 6 and 9.

* * * * * * * * *

Applicants hereby request a one-month extension to respond to the February 22, 2006 Office action, and authorize the Commissioner to charge Deposit Account No. **02-2334** for the corresponding extension fee. In addition, Applicants are filing this Amendment D with a request for continued examination, and authorize the Commissioner to charge Deposit Account No. **02-2334** for the fee corresponding to that request. Applicants do not believe that they owe any other fee in connection with this filing. If, however, Applicants do owe any such fee(s), the Commissioner is hereby authorized to charge the fee(s) to Deposit Account No. **02-2334**. In addition, if there is ever any other fee deficiency or overpayment under 37 C.F.R. §1.16 or 1.17 in connection with this patent application, the Commissioner is hereby authorized to charge such deficiency or overpayment to Deposit Account No. **02-2334**.

Applicants submit that the pending claims are in condition for allowance, and request that this application be allowed. The Examiner is requested to call the Undersigned if any issues arise that can be addressed over the phone to expedite examination of this application.

Respectfully submitted,



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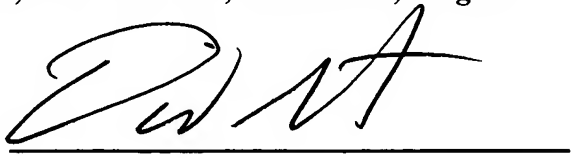
CERTIFICATE OF MAILING UNDER 37 CFR §1.8

I certify that this correspondence is being deposited with the U.S. Postal Service on **June 22, 2006** with sufficient postage as first class mail to **Mail Stop RCE, Attn: Examiner Dr. Sumesh Kaushal, Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450.**



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enclosures

(12) PATENT APPLICATION (11) Application No. **AU 198285929 A1**
(19) AUSTRALIAN PATENT OFFICE

(54) Title
MASTITIS VACCINE

(51) International Patent Classification(s)
A61K 039/085

(21) Application No: **198285929** (22) Date of Filing: **1982.07.12**

(30) Priority Data

(31) Number	(32) Date	(33) Country
PE9718	**1981.07.14**	**AU**

(43) Publication Journal Date: **1983.01.20**

(71) Applicant(s)
Commonwealth Scientific and Industrial Research Organisation

(54) Inventor(s)
Name not given

85929/82

FORM 1

COMMONWEALTH OF AUSTRALIA

LODGE | AUSTRALIAN | 15 JUL 1981 | PATENT OFFICE

THE PATENTS ACT 1952-1973

APPLICATION FOR A PATENT

COMPLETE AFTER PROVISIONAL SPECIFICATION No. 85924/82

We, COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANIZATION, of Limestone Avenue, Campbell, ACT, hereby apply for the grant of a Patent for an invention entitled:

"MASTITIS VACCINE"

which is described in the accompanying provisional specification.

RECEIVED
Date 14.7.81
Receipt PE9718
Application ✓
Declaration —
Specification 9+7C
Drawings —

Our address for service is:

C/- The Director
Bureau of Scientific Services
CSIRO Head Office
Limestone Avenue
CAMPBELL ACT 2601

(Postal address: PO Box 225
DICKSON ACT 2602)

DATED this 14th day of July 1981.

COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANIZATION

By: G. Burgess
Registered Patent Attorney

AUSTRALIAN

FEE STAMP TO VALUE OF $ 142 ATTACHED
MAIL OFFICER

TO:
THE COMMISSIONER OF PATENTS

(12) AUSTRALIAN PATENT ABSTRACT
(19) AU (11) AU-A-85 929/82

(54) MASTITIS VACCINE

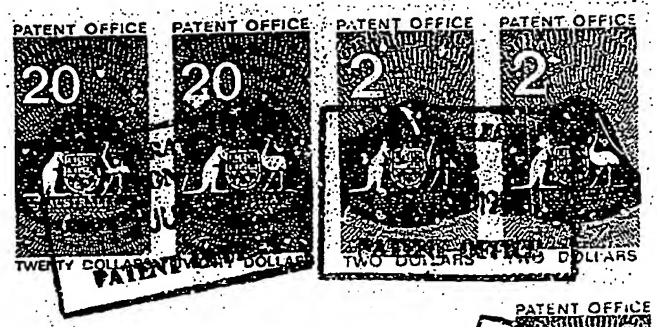
(71) COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANIZATION

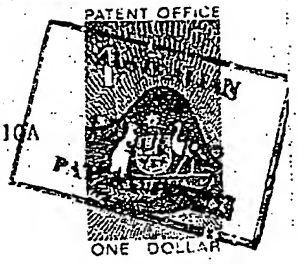
(21) 85 929/82 (22) 14.7.81 (23) 12.7.82 (24) 14.7.81
(43) 20.1.83
(51)[3] A61K 39/085
(72) NOT GIVEN

(57) Claim

1. A vaccine effective in the immunisation of ruminants against intermammary challenge by homologous or heterologous strains of S.aureus.

3. A method as claimed in claim 1 in which the strain of S. aureus is a catalase and coagulase - positive strain of ruminant origin.

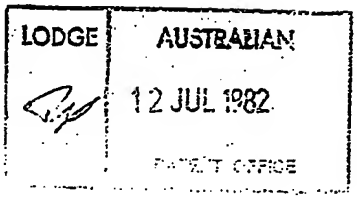




Form 10A

COMMONWEALTH OF AUSTRALIA

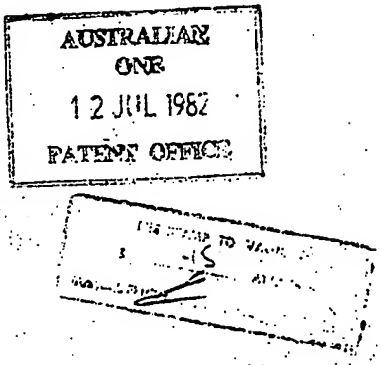
Patents Act 1952



COMPLETE PATENT SPECIFICATION FOR THE INVENTION ENTITLED

"MASTITIS VACCINE"

The following is a full description of this invention, including the best method of performing it known to me:



This invention concerns the immunisation of ruminants against staphlococcal mastitis.

Staphylococcal mastitis is a disease condition affecting approximately 25% of Australian dairy cattle, which is estimated to cost the industry about $30,000,000 each year in lost milk production, culling, antibiotics and costly husbandry procedures.

Traditionally the disease is treated, on the appearance of clinical symptoms, by the infusion of antibiotics which are active against the infective organism, Staphyloccoccus aureus. Such an approach is far from ideal. Apart from being curative rather than preventative, there are economic considerations such as the need for identification and individual treatment of affected animals and the unsuitability of antibiotic-contaminated milk for human consumption or processing. An additional problem is the increasing development of antibiotic-resistant strains of S. aureus. Attention has, therefore, turned towards the possibility of immunizing animals against the infection. Until now, however, there has been no vaccine which will provide resistance to homologous or heterologous strains of S. aureus. The reasons for this are imperfectly understood. However, as we have now developed a technique for preparing a live vaccine which is capable of conferring a useful level of homologous and heterologous immunity, it is thought that previous vaccines, which were dead vaccines, lacked certain essential virulence determinants produced only under in vivo growth conditions.

Accordingly, in its broadest aspect this invention provides a vaccine effective in the immunisation of ruminants

against intramammary challenge by homologous or heterologous strains of S. aureus.

In another aspect the invention provides a method of preparing an effective and safe staphylococcal mastitis vaccine. This method is characterised by multiple passage of a strain of S. aureus until loss of haemolytic activity is noted.

In general terms the vaccine may be prepared by attenuating a catalase and coagulase - positive strain of S. aureus, of ruminant origin, by multiple passage on a suitable bacteriological growth media until loss of haemolytic activity is achieved. The attenuated strain may then be cultured in a suitable bacteriological media, washed free of the media and exotoxins with a non-bacterio-static diluent, and, using the same diluent, diluted to the desired concentration (at least about 10^8 S. aureus/ml.) for administration.

The following is an example of the preparation of an S. aureus vaccine by the method of this invention and of animal trials embracing use of the vaccine:

EXAMPLE

Preparation

A catalase and coagulase positive strain of Staphylococcus aureus (Strain WA 79) was isolated from an acute case of bovine mastitis. It was attentuated by multiple passage on 5% sheep blood agar until haemolytic activity could no longer be observed, after which the strain was maintained on mannitol-salt agar sloped at 4°C. When required, a vaccine was prepared by growing the bacteria in nutrient broth (Oxoi CM1) for 24 hours at 37°C with shaking; the culture

was then washed twice with sterile phosphate-buffered saline (PBS) and adjusted to a concentration of 10^{10}/ml with sterile PBS.

Experimental Procedure

12 pregnant primiparous Merino ewes were maintained in phalaris-white cover pastures with water provided ad libitum. Each ewe was allowed to rear its lamb. The animals were randomly allocated to two treatment groups (Table 1). Ewes in Group 1 were given 1 ml of the vaccine subcutaneously in the left hindleg at 6 weeks and 4 weeks prepartum. Ewes in Group 2 were maintained as a non-vaccinated control.

Serum was prepared from blood samples collected at intervals before and after vaccination. Whey was prepared by centrifugation of milk samples collected from each animal immediately prior to challenge and both serum and whey were stored at -16°C until required for assay.

At 30-35 days post-partum both mammary glands of ewes in both groups were challenged by infusion of 1 ml of viable S. aureus (Strain Wood 46) at a concentration of 10^6/ml. Lambs were removed from the ewes for the 6 hours after challenge. Clinical assessments, milk production measurements, leucocyte counts and assays of bacteriological status were made before and at intervals after challenge.

Results

Antibody Responses. Results for titres of anti-staphylococcal agglutinating antibody in blood serum and whey are presented in Table 1. Moderate increases in serum titres were recorded for animals in Group 1, but there was no significant difference

between treatment groups for titres in milk whey immediately prior to challenge.

Opsonisation Assays

The radioisotope technique of Lam & Mathieson (1979 - J.med. Microbiol. 12:459) was used to compare the opsonizing capacity of serum and whey from ewes in each treatment group. The effector cells were neutrophils obtained from involuted mammary glands of multiparous, non-immunised ewes and an incubation period of 30 minutes was allowed. The opsonisation index is computed as equivalent to the percentage of inhibition, due to internalisation, of radioisotope uptake by the cccci. It therefore provides a direct measure of internalisation of cocci by neutrophils. There was no significant difference between the groups for indices for serum collected at the time of the primary and booster vaccinations, however by 5 weeks post-partum the mean index for the serum of animals of Group 1 was significantly greater (62 cf.52) than that for Group 2. There was no significant difference between the opsonisation indices for milk whey at that time.

Clinical Response after Challenge

The numbers of ewes in each group exhibiting clinical signs of acute mastitis are shown in Table 2. In these animals the mammary glands were severely swollen and oedematous; there was depression and pyrexia with rectal temperatures for control ewes reaching 40.4± 0.4°C at 24 hours post challenge.

The proportions of ewes in each group shedding S. aureus in milk in the 48 hours following challenge are also shown in Table 2. It can be seen that there was a marked reduction

in the proportion of vaccinated ewes which shed the bacteria over this period.

Luecocyte Count

Results for leucocyte counts in milk after challenge are shown in Table 3. Significant leucocytosis developed in all animals, with maximal values occurring 24 hours after infection. However, at 8 days post-challenge the mean value for the control group was significantly greater than for the vaccinated group.

Milk Production

Data for milk production post-challenge is presented in Fig.1. On day 3 the mean production for ewes of the vaccinated group was significantly better than for the control of ewes and remained so for at least a further 5 days.

The experiments demonstrated that ewes immunised with a vaccine according to this invention developed a considerable degree of immune resistance to challenge with a heterologous strain. On the basis of clinical criteria and changes in milk production, following challenge, the vaccine was considered to provide a useful measure of protection.

Vaccines according to this invention may be administered subcutaneously or intradermally typically (at a concentration of 10^{10}/m) as two doses of 1 ml each at least two weeks apart.

CLAIMS

The claims defining the invention are as follows:

1. A vaccine effective in the immunisation of ruminants against intermammary challenge by homologous or heterologous strains of S.aureus.

2. A method of preparing a vaccine as claimed in claim 1 which comprises the multiple passage of a strain of S. aureus until loss of haemolytic activity is observed.

3. A method as claimed in claim 1 in which the strain of S. aureus is a catalase and coagulase - positive strain of ruminant origin.

4. A method as claimed in either claim 2 or 3 in which, after multiple passage, the attenuated strain is cultured in a suitable bacteriological media, washed free of the media and exotoxins with a non-bacteriostatic diluent and diluted to the desired concentration with the same diluent.

5. A method as claimed in claim 4 in which the desired concentration is at least 10^8 S. aureus/ml.

6. A method of preparing a vaccine as claimed in claim 1, substantially as described in the Example herein.

7. A vaccine as claimed in claim 1 when prepared by the method as claimed in any one of claims 2-6.

Dated this 12th day of July, 1982.

COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANIZATION

TABLE 1

Anti-staphlococcal Agglutination Titres (Log_2) in Blood Serum and Milk They

Treatment Group	Time Before or After Parturition (Weeks)				
	-6 Serum	-4 Serum	0 Serum	+5 Serum	+5 They
1	4.7±0.4	5.7±0.4	7.3±0.3	8.0±0.5	0.4±0.3
2	4.6±0.5	5.2±0.3	3.8±0.2	3.6±0.5	1.2±0.3

Values presented are means ± standard errors

TABLE 2

Proportions of Ewes in Each Treatment Group Exhibiting Clinical Signs of Actue Mastitis and Schedding S. aureus in Milk after Challenge

Treatment Group	Clinical Signs of Acute Mastitis			Shedding S. aureus in Milk		
	Time after challenge (hours)					
	6	24	48	6	24	48
1	1	1	1	7	5	3
2	3	4	4	5	5	4

Total number of animals in Group 1 = 7
Total number of animals in Group 2 = 5

TABLE 3

Leucocyte Counts (log_{10}) per ml before and at intervals after Challenge

Treatment Group	Time after Challenge (hours)				
	0	6	24	48	192
1	5.07±0.05	6.53±0.10	7.16±0.14	6.34±0.17	6.08±0.30
2	4.98±0.15	7.35±0.07	7.82±0.12	7.22±0.35	7.45±0.28

Values are means ± standard errors

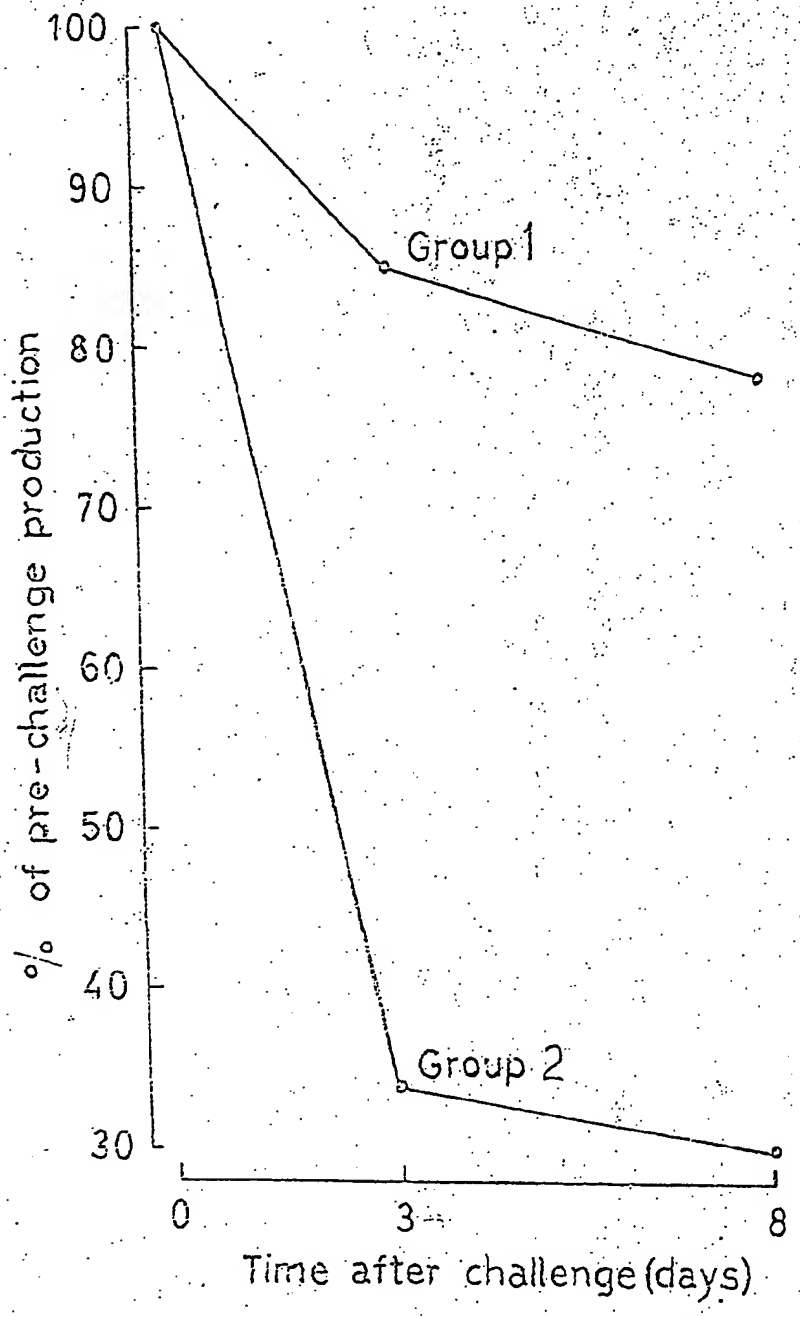


Figure 1. Milk production data for ewes in the 2 treatment groups Values presented are means of post-challenge production expressed as percentages of pre-challenge productions.

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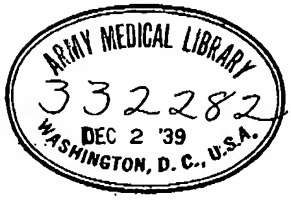

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BOVINE CONTAGIOUS ABORTION.

THE USE OF GUINEA-PIGS IN IMMUNISATION STUDIES.

By A. D. McEWEN and R. S. ROBERTS,

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THE few records of immunisation experiments against *Br. abortus* infection in guinea-pigs are insufficient to warrant any conclusions regarding the value of these animals for this type of work. Experiments with killed cultures have been recorded by Ascoli (1916), Stafseth (1920), Hagan (1922) and Gwatkins (1931). Ascoli and Stafseth concluded that no immunity was produced, but their methods of testing immunity were most probably too severe. Hagan paid adequate attention to experimental details but the numbers of animals used were small, only seven vaccinated animals being available for comparison with seven controls; he considered, however, that *Br. abortus* infection developed more slowly in the vaccinated animals. Gwatkins used repeated large inoculations of killed cultures but no immunity was demonstrated.

More significant results are reported from the inoculation of living cultures of low virulence. Huddleson (1924) stated that a living culture of a non-virulent strain of *Br. abortus* had been found to protect guinea-pigs from abortion, when exposed to infection by feeding the animals with virulent micro-organisms, but no details of his experiments are given. Schroeder and Cotton (1925) reported upon the inoculation of guinea-pigs with a strain which only caused lesions when very large doses were given. The strain, however, was recoverable from the spleens of guinea-pigs two months after their inoculation with moderate doses. They vaccinated guinea-pigs with heavy suspensions of this living culture and exposed some of these animals and control guinea-pigs to a subcutaneous infection with a bovine strain of *Br. abortus*; the other vaccinated animals, together with controls, were infected with a porcine strain of *Br. abortus*. The quantities of these

respective test inoculations are not given, but, on the whole, when the animals were killed and examined the vaccinated guinea-pigs showed fewer lesions or less extensive lesions than the controls. No bacteriological examinations were made.

Were it possible to increase the resistance of both guinea-pigs and cattle by the use of the same vaccine, the guinea-pig might prove a most valuable laboratory animal for the routine examination of vaccines used in the field.

The primary objective of the experiments now to be summarised was to ascertain whether a strain of *Br. abortus* of low virulence stimulated a measurable resistance to a subsequent exposure to an infection with a virulent strain of *Br. abortus*. Only one strain of low virulence has been tested, Strain No. 45. This strain was isolated some years ago in this country from material of bovine origin, most probably prior to 1922, but the exact date of its isolation is uncertain. It behaves as a typical bovine strain; it is agglutinated well by positive sera, and throughout the course of these experiments it has remained smooth, being heat-stable according to the criteria adopted by Pandit and Wilson (1932). Comparative tests have shown that the strain grows equally as well in ordinary atmospheric air as in an atmosphere containing 10 per cent. CO_2 gas, irrespective of whether the seed material is derived from a stock culture or from the tissues of an inoculated animal.

In 1931 a few preliminary experiments demonstrated the low virulence of the strain for guinea-pigs and suggested the possibility of its possessing antigenic value, and in September 1931 the current sub-culture of that date was arbitrarily designated Sub-culture X1, and sub-cultures from that time have been numbered in series.

Since March 1930, Strain 45 has been maintained exclusively on liver agar slants. The virulent strains used in testing the resistance or immunity of guinea-pigs have been grown exclusively on the same medium. All suspensions of the organisms used in the preparation of vaccine and for the test inoculations, etc., have been prepared from 48-hour growths on liver agar, washed off with normal saline solution and standardised to a required opacity, using Brown's opacity tubes for this purpose.

On a table supplied with the opacity tubes, the numbers of organisms in suspensions of *Br. melitensis* of opacity comparable to those of the different tubes in the set are given. It has been arbitrarily assumed that suspensions of *Br. abortus* contain a like number of micro-organisms to comparable suspensions of *Br. melitensis*. On this basis and accepting the numbers of *Br. melitensis* given in the above-mentioned table as correct, so in the succeeding records the numbers of *Br. abortus* in the different suspensions used are given, the reference to the actual opacity of the suspensions generally being omitted. Suspensions for the

agglutination test have been prepared either from Strain 45 or from another smooth strain. The same suspension has always been used throughout any one experiment.

In order to ensure a CO_2 sensitivity in the strain used to test for immunity it has been necessary to use more than one CO_2 sensitive strain throughout the experiments. In one experiment vaccinated animals were deliberately infected with a virulent ærobic strain.

Prior to 1933, when the experimental guinea-pigs were killed and examined, the spleen was removed and portions of the organ thoroughly teased with needles; a loopful of spleen tissue was then inoculated on to agar slants. When other tissues were examined for evidence of infection these were treated in a similar manner. From 1933 onwards the spleens were weighed and afterwards reduced to a pulp by scraping with a spatula; a quantity of the pulp was reserved for the inoculation of agar slants and the remainder of the pulp transferred to sterile thick-walled test tubes containing a number of glass beads, a measured volume of sterile saline solution was then added, the tube closed with a rubber stopper and vigorously shaken. A 1 : 10 dilution of the spleen pulp suspension was made in a second tube. From these two suspensions, amounts of 0·2 c.c. were sown on fuchsin agar plates, or on agar slants in large test tubes of 1 in. internal diameter and from the number of colonies which formed the number which theoretically could have been obtained from the whole spleen was estimated. From comparisons made on cultures from a large number of guinea-pigs, the enumeration of colonies obtained from spleen emulsions provided no more reliable data than that obtained by the direct inoculation of spleen pulp on agar.

When material from animals vaccinated with living cultures of Strain 45 was examined the absence of growth in atmospheric air was regarded as evidence of the elimination of the vaccine strain from the body.

Attempts have been made to passage Strain 45 through guinea-pigs. It was found impossible to passage the strain directly from animal to animal by the inoculation of spleen tissue, the strain invariably being lost in the second passage animal. The strain, however, was generally recoverable from the spleens of guinea-pigs inoculated seven to ten days previously with large numbers of bacteria, but growth was not abundant, only a few colonies being obtained per slant richly seeded with spleen pulp; sub-cultivation of these primary growths yielded rich cultures which were in turn inoculated into guinea-pigs, and so on. From February to May 1935, the strain was passaged through a series of six guinea-pigs by such means.

When Strain 45 was tested for pathogenicity in 1931 it was found that the subcutaneous or intraperitoneal inoculation of guinea-pigs each with 768 million micro-organisms or the

subcutaneous inoculation of twice that number failed to produce any macroscopic evidence of infection or to produce agglutinins in titres higher than 1 : 160. However, from the spleens of some animals a few colonies of *Br. abortus* were obtained even up to 33 days after inoculation. By June of 1934 such *in vivo* survival powers as the strain may have possessed three years previously had probably diminished, because the micro-organism was not recoverable after the fourth week in any one instance from the spleens of guinea-pigs inoculated subcutaneously with the relatively enormous dose of 61,480 million micro-organisms. The titre of the serum remained low, even after these large inoculations. In one experiment where 20 guinea-pigs were used, the serum from one animal agglutinated at a titre of 1 : 640 and the serum from another at 1 : 320, but the remaining sera all had lower titres.

The vaccination experiments of 1931 further indicated the absence of invasive properties of the strain even at that time, no evidence having been obtained from the examination of guinea-pigs in immunisation experiments of the persistence of the strain in the body of the guinea-pigs a few weeks after inoculation.

The conclusion that the strain was of low virulence for the guinea-pigs throughout the four years under consideration is justifiable. Furthermore, from the protocols of the last vaccination experiment which are summarised later, it is apparent that even after serial passage through six guinea-pigs the virulence of the passaged strain had not been exalted to an appreciable extent, if at all.

Immunisation Experiments.

1931-32 *Experiments.*

Preliminary experiments made in 1931 indicated that guinea-pigs inoculated once with 1,537 million living micro-organisms of Strain 45 possessed greater resistance to infection caused by applying a drop of a suspension of a recently isolated CO_2 sensitive strain of *Br. abortus* containing 2,305 million organisms per cubic centimetre, to the eye, than control animals. Furthermore, the inoculation of three doses of living micro-organisms in ascending magnitude at approximately weekly intervals, stimulated an appreciable resistance in a group of guinea-pigs which included a number of female animals; and a comparison of pregnant vaccinated animals with pregnant control animals showed a greater susceptibility to uterine infection and abortion among the latter. The experiments of that year, however, indicated that when a number of guinea-pigs were inoculated with the same living vaccine and divided into two groups and later infected in a comparable manner but on different dates, the apparent resistance to infection of the groups might differ.

An opportunity to carry out further experiments did not occur until September 1932, when eleven male guinea-pigs each received

three subcutaneous inoculations, each consisting of 1,537 million organisms of Strain 45 Sub-culture X5. Fifty-nine days after receiving the final inoculation the vaccinated animals and an equal number of control male guinea-pigs received subcutaneously a test inoculation of a virulent CO_2 sensitive strain of *Br. abortus*. The test inoculation consisted of 1 c.c. of a suspension of organisms equivalent in opacity to No. 1 tube of the Brown scale (containing 768 million organisms per cubic centimetre) diluted 10^7. The dilutions, however, were all made with the same pipette, and therefore the approximate number of organisms inoculated cannot be even roughly estimated. Equal numbers of the vaccinated and control animals were killed and examined later at intervals of 43, 57, 60 and 71 days respectively. None of the vaccinated animals showed lesions, whereas the characteristic splenic enlargement of a *Br. abortus* infection was shown by all the controls, and two of these animals, one killed on the forty-third day and the other on the sixtieth day, each showed abscessation of one testicle. *Br. abortus* was recovered in culture from the spleen of one vaccinated guinea-pig but was not obtained from the livers of any of the animals. *Br. abortus* was, on the other hand, recovered in culture from the spleens of all the control animals and from the livers of four of them. The titres of the sera when the animals were killed further reflected the decided resistance of the vaccinated animals to infection. The titres of vaccinated animals were 1 : 40, 1 : 80, 1 : 80, 1 : 40, 0, 1 : 80, 1 : 320, 0, 1 : 160, 0 and 1 : 80, and those of the control animals 1 : 640, 1 : 2,560, 1 : 2,560, 1 : 1,280, 1 : 10,240, 1 : 2,560, 1 : 640, 1 : 640, 1 : 80, 1 : 640 and 1 : 2,560.*

The results of this experiment were unequivocally in favour of the vaccinated group which showed, according to the criteria of the examination, a complete resistance in 90 per cent. of the animals to an exposure which caused infection in 100 per cent of the controls.

1933—*Experiment on Male Guinea-pigs.*

The next experiment was commenced in May 1933 and it was designed in the hope of repeating the favourable results of the preceding experiment: and also to try to compare the immunising properties of vaccines consisting of living organisms of Strain 45 Sub-culture X226, obtained by repeated sub-cultivation at intervals of one to two days; of heat killed suspensions of Strain 45 Sub-culture X9; and of formalin sterilised suspensions of the same strain and sub-culture, with suspensions of live organisms of the same.

Male guinea-pigs were used and vaccinated three times at intervals of 14 days. The animals given the living suspensions of *Br. abortus* received 3,074 million organisms at each inoculation;

* 0 = No agglutination at 1 : 20.

those receiving suspensions sterilised by heat and formalin respectively, 30,740 million organisms at each inoculation. The sterilisation of the former was carried out by heating in the water bath at 65° C. for one hour; the sterilisation by formalin was accomplished by adding sufficient formalin to the suspension of the organisms to give a final concentration of 0·5 per cent. formalin and incubating at 37° C. for 48 hours.

The vaccinated guinea-pigs and control animals each received a test inoculation 14 days after the administration of the last inoculation of vaccine. The test inoculation was similar to that employed in the preceding experiment of September 1932, except in so far as the sixth instead of the fourth sub-culture of the CO_2 sensitive strain was used. The guinea-pigs were killed and examined 31, 34, 35, 39, 40, 45, 48 and 49 days after infection, as far as possible equal numbers being killed from each group at the same time.

The results of this experiment were not very satisfactory and may be summarised as follows: Of 15 animals inoculated with live vaccine from Sub-culture X9, eleven, or 73 per cent., were infected, and one, or 6 per cent., showed macroscopic lesions; of 13 animals inoculated with live vaccine from Sub-culture X226, ten, or 77 per cent., were found to be infected, and two, or 15 per cent., showed macroscopic lesions; of 15 animals inoculated with heat killed vaccine, fourteen, or 93 per cent., were infected, and eight, or 53 per cent., showed macroscopic lesions; of 15 inoculated with formalinised vaccine, twelve, or 80 per cent., were infected, and four, or 26 per cent., showed macroscopic lesions; 16 out of 16 controls were infected, and six, or 37 per cent., showed macroscopic lesions.

The experiment, though of limited value, does not suggest that the repeated sub-cultivation had depleted Strain 45 of antigenic properties and it indicates that heat killed vaccine may be of little value.

In a further experiment in 1933 comparisons were made between male guinea-pigs inoculated four times with 61,840 million live organisms at intervals of one week and similar animals inoculated in the same manner but with a number of micro-organisms ten times smaller. The animals were infected six weeks after the last inoculation of vaccine. Owing to intercurrent disease the numbers of the animals in experiment were considerably reduced but the summarised results in Table I show that vaccination with either the large or the smaller dose had increased the animals' resistance to infection.

1934—*Experiments on Male Guinea-pigs.*

An experiment was commenced in May 1934, and was designed to test the effect of large multiple doses of vaccine; multiple smaller sized doses; a single large dose and single smaller dose;

TABLE I.

No. of G. Pigs per group.	No. and Percentage found infected.	No. and Percentage showing Macroscopic Lesions.	Average No. of Colonies from Spleen Cultures, Infected Animals only considered.	No. and Percentage with infected Livers.	Average No. of Colonies from Liver Cultures.	Average Titre of Serum.

TABLE I.

	No. of G. Pigs per group.	No. and Percentage found infected.	No. and Percentage showing Macroscopic Lesions.	Average No. of Colonies from Spleen Cultures, Infected Animals only considered.	No. and Percentage with infected Livers.	Average No. of Colonies from Liver Cultures.	Average Titre of Serum.
Group 1. Large multiple inoculations	10	2 20 per cent.	0 0 per cent.	7	0 0 per cent.	0	25
Group 2. Moderate multiple inoculations	6	2 33 per cent.	0 0 per cent.	8	0 0 per cent.	0	38
Group 5. Controls ...	8	8 100 per cent.	8 100 per cent.	104	3 37 per cent.	3	640

In estimating the average titre of the serum, the dilution of the serum at the end point of agglutination was regarded as a fraction.

TABLE II.

PLAN OF EXPERIMENT OF MAY, 1934.

	Weeks.												
Group of Guinea Pigs.	1st.	2nd.	3rd.	4th.	5th.	6th.	7th.	8th.	9th.	10th.	15th.	20th.	25th.
1 ...	10uk.	10uk.	10uk.	10uk.						Inf.	Ex.		
2 ...	1ul.	1ul.	1ul.	1ul.						„	„		
3 ...	10ul.	10ul.	10ul.	10ul.						„	„		
4 ...				1ul.						„	„		
5 ...				0·1ul.						„	„		
6 ...						1ul.	1ul.	1ul.	1ul.	„	„		
7 ...						10ul.	10ul.	10ul.	10ul.	„	„		
8 ...									1ul.	„	„		
9 ...									10ul.	„	„		
10 ...										„	„		
11 ...		1ul.	1ul.	1ul.	1ul.							Inf.	Ex.
12 ...		10ul.	10ul.	10ul.	10ul.							„	„
13 ...												„	„

10uk. = 10 units of killed vaccine, one unit containing 6,148 million micro-organisms.
1ul. = 1 unit of living vaccine, one unit containing 6,148 million micro-organisms.
10ul. = 10 units of living vaccine, one unit containing 6,148 million micro-organisms.
Inf. = Infected with 23,000 organisms of a virulent CO_2 sensitive strain of *Br. abortus*. In preparing these and all subsequent test inoculations a separate pipette was used in making each dilution.
Ex. = Killed and examined.

TABLE III.

Group and Vaccine.	Dose of Vaccine.	Time in Weeks before Infection.	No. of G. Pigs per Group.	No. and Percentage Infected.	No. and Percentage Heavily Infected in Spleen.	No. and Percentage Infected in Liver.	Average Weights of Spleens.	No. of Sera with a Titre of 1 : 10 and Higher and Average Titre of these Sera.
1. Heat killed	L.M.	6	15	12	6	1	1·08	14
				80 per cent.	40 per cent.	6·6 per cent.		56
2. Living	S.M.	6	17	1	0	0	0·77	16
				5·9 per cent.	0 per cent.	0 per cent.		32
3. Living	L.M.	6	16	4	0	0	0·84	13
				25 per cent.	0 per cent.	0 per cent.		29
4. Living	S.S.	6	18	3	0	0	1·00	14
				16·6 per cent.	0 per cent.	0 per cent.		34
5. Living	L.S.	6	18	3	0	0	0·85	17
				16·6 per cent.	0 per cent.	0 per cent.		41
6. Living	S.M.	1	18	18	2	1	0·97	18
				100 per cent.	11 per cent.	5·5 per cent.		134
7. Living	L.M.	1	17	15	1	1	0·89	16
				88·2 per cent.	5·8 per cent.	5·8 per cent.		117
8. Living	S.S.	1	17	14	1	1	0·89	16
				82·3 per cent.	5·8 per cent.	5·8 per cent.		50
9. Living	L.S.	1	17	12	3	3	0·93	17
				70·5 per cent.	17·4 per cent.	17·4 per cent.		45
10. Controls	—	—	18	18	8	7	1·36	18
				100 per cent.	44 per cent.	38·5 per cent.		147
11. Living	S.M.	15	18	17	2	3	1·45	18
				94 per cent.	11·5 per cent.	16·5 per cent.		241
12. Living	L.M.	15	16	13	2	2	1·11	16
				71·1 per cent.	12·4 per cent.	2·4 per cent.		106
13. Controls	—	—	18	18	6	8	1·88	18
				100 per cent.	33·3 per cent.	44 per cent.		461

Aerobic strains of *Br. abortus* were isolated from five animals in each of Groups 6 and 7 and from 6 animals in each of Groups 8 and 9 after vaccination, and its isolation from the animals in these groups must be attributed to the effect of inoculating the animals with a virulent culture shortly after they had been vaccinated.

L.M. = large multiple : S.M. = small multiple : L.S. = large single : S.S. = small single.

and also to test the immunity at different periods of time after the last inoculation of vaccine. Furthermore, it was planned to test a vaccine composed of dead organisms sterilised by heat. The living vaccine was in each case prepared from Strain 45 Sub-culture X14; the dead vaccine was prepared from the same strain Sub-culture X13. The vaccine sterilised by heat had been maintained at a temperature of 60° C. for one hour, no preservative was added, and the vaccine was kept in the ice chest.

The plan of the experiment and its results are given in Tables II and III.

From the above summary it is evident that where the living vaccine was used satisfactory results were obtained with either multiple or single inoculations of large or smaller doses of vaccine when the test inoculation was given six weeks after the last inoculation of vaccine, the best results being shown by Groups 2, 3, 4 and 5. When the results of these groups are considered together they show that out of 69 guinea-pigs 58, or 84 per cent., completely resisted an infection which was sufficient to infect all of the 18 control animals.

When the test inoculation was given one week after the inoculation of the last dose of vaccine the results were not satisfactory, but in none of these vaccinated groups is there any evidence that the inoculation, even of large quantities of the vaccine strain only one week before infection, had increased the susceptibility to infection. Indeed, the evidence indicates some slight increase in resistance, as nine out of 69 guinea-pigs in Groups 6, 7, 8 and 9 resisted infection. Furthermore, in some of the remaining 60 guinea-pigs it is probable that the infection which was found was due to a persistence of the vaccine strain.

In this experiment those animals which received one inoculation only of vaccine, Groups 8 and 9, and could have developed no immunity from previous inoculations of vaccine, were significantly no more susceptible than the controls, Group 10, although viable organisms from the vaccine must have been present in the bodies of the vaccinated animals when the test inoculation was administered.

In that portion of the experiment where the resistance of the animals was tested 15 weeks after the last inoculation of vaccine the results are interesting. Although the animals in Groups 11 and 12 were but very little more resistant than the controls, they had been inoculated with comparable and in some instances with exactly the same vaccine which had produced a very definite immunity in the majority of the guinea-pigs in Groups 2, 3, 4 and 5. Therefore the failure to demonstrate a definitely increased resistance in the animals in Groups 11 and 12 cannot be attributed to any variation in the vaccine. Groups 11 and 12 were infected 15 weeks after vaccination, whereas Groups 2, 3, 4 and 5 were exposed to infection only six weeks after vaccination, but it is

most unlikely that a detectable diminution in immunity could occur between the seventh and the sixteenth week following vaccination. Moreover, in a later experiment immunity was shown to persist for at least 19 weeks. In the present experiment the same virulent strain of *Br. abortus* was used in the preparation of the test inoculations for all the groups of guinea-pigs, and when test inoculations were prepared comparable methods were employed in standardising the suspensions of culture to a like capacity and in the preparation of the requisite dilutions. Nevertheless it is probable that the inconstant results of this and of some other experiments were due to a variation in the severity of the test inoculations used and that these variations were uncontrollable by the methods employed.

The object of the next experiment was to test the effect of exposing vaccinated guinea-pigs to an infection with a virulent ærobic strain of *Br. abortus* and later exposing the animals to a second infection, but with a CO_2 sensitive strain of *Br. abortus*. Furthermore, the experiment was so arranged that it might furnish some data upon the duration of increased resistance in guinea-pigs.

Three groups of vaccinated and three groups of control guinea-pigs were used. The vaccinated groups are designated V.1, V.2 and V.3, and the control groups C.1, C.2 and C.3. All the animals in the vaccinated groups were inoculated at the same time and with the same vaccine, each animal receiving one inoculation of 6,148 million live organisms of Strain 45, Sub-culture X13.

Eight weeks after vaccination the animals in Groups V.1 and V.2, and the control guinea-pigs in Group C.1, were each inoculated with 30,700 organisms of a virulent ærobic strain of *Br. abortus*. Nineteen weeks after receiving this test inoculation the animals in Groups V.1 and C.1 were killed and examined. *Br. abortus* was isolated from three of the vaccinated animals, in two instances in very small numbers only, and from eight of the ten control guinea-pigs, thereby indicating that the vaccinated animals had possessed an increased resistance to infection. The guinea-pigs in Groups V.3 and C.3 were each inoculated with 30,700 organisms of a CO_2 sensitive strain 19 weeks after the animals in Group V.3 had been vaccinated. Seven weeks later the animals in both of these groups were killed and examined, that is on the twenty-sixth week of the experiment. Of the nine surviving vaccinated animals none was found infected with *Br. abortus*, whereas this micro-organism was recovered from eight out of ten animals in the control group. This portion of the experiment therefore furnished evidence that an immunity produced by vaccination was present 19 weeks after vaccination. The vaccinated animals in Group V.2 which had been inoculated eight weeks after vaccination with the virulent ærobic strain of *Br. abortus* were each inoculated 18 weeks later, that is 26 weeks after vaccina-

tion, with 30,700 organisms of the CO_2 sensitive strain previously used for the inoculation of Groups V.3 and C.3; the control animals in Group C.2 were similarly inoculated at this time. Six weeks later the animals in Groups V.2 and C.2 were killed and examined. *Br. abortus* was recovered from three of the nine surviving animals in Group V.2 and in each instance the recorded organisms were ærobic. All of the eight surviving control animals in Group C.2 showed a heavy infection with a CO_2 sensitive strain of *Br. abortus*.

In considering the results obtained in the six groups it appears that in Groups V.1 and C.1 the vaccination had produced a partial immunity to the inoculation of the ærobic strain of *Br. abortus* eight weeks later, and in Groups V.3 and C.3 a definite immunity to the inoculation of the CO_2 sensitive strain 19 weeks after vaccination. Furthermore, that vaccinated guinea-pigs exposed to infection with the ærobic strain, Group V.2, were able to withstand a later exposure to a CO_2 sensitive strain although certain of the guinea-pigs were at the time actually infected. The resistance of these guinea-pigs to the superimposition of infection is of particular interest and the experiment indicates the feasibility of testing the immunity produced by exposure to ærobic strains of some degree of pathogenicity, either in previously vaccinated animals or in normal animals, when the final test for increased resistance is carried out with a CO_2 sensitive strain. Finally, the experiment showed that an immunity response to the inoculation of the non-virulent Strain 45 persisted for a period of at least 19 weeks.

A summary of the experiment is given in Table IV.

TABLE IV.

Groups of G. Pigs.	*Weeks of Experiment.*				*No. of G. Pigs Examined.*	*Percentage found Infected.*
	8th.	*19th.*	*26th.*	*32nd.*		
Vaccinated 1	Inf. with ærobic	Examine			10	30 with ærobic.
Control 1	„	„			10	80 with ærobic.
Vaccinated 2	„		Inf. with CO_2	Examine	9	33 with ærobic only.
Control 2			„	„	8	100 with CO_2.
Vaccinated 3		Inf. with CO_2	Examine		9	None.
Control 3		„	„		10	80 with CO_2.

1934—*Experiments on Female Guinea-pigs.*

It had been noted during 1931 that infected pregnant female guinea-pigs showed a heavy *Br. abortus* infection of the uterus; and that vaccinated pregnant guinea-pigs after exposure to infection, though sometimes infected in the spleen, might be free from

a uterine infection, or less severely infected in that organ than comparable control animals. If by vaccination with a non-virulent strain of *Br. abortus* it were possible to increase the resistance of the pregnant uterus to infection, even although the immunity were insufficient to afford the guinea-pig complete protection against infection, it would encourage the hope that the comparable treatment of the bovine might give equally favourable results in the protection of the pregnant uterus.

In the experiment about to be described female guinea-pigs were used and were infected after mating. Although abortion in infected guinea-pigs has been observed to occur with considerable regularity, nevertheless it was difficult to detect in individuals kept in groups, therefore reliance had to be placed on evidence of uterine infection at *post-mortem* examination, and the guinea-pigs were killed when it was considered most likely to find the majority of the animals pregnant or to have but recently aborted or given birth to young. Infection of the pregnant uterus was demonstrated by inoculating portions of the placentæ on to liver agar slopes. In cases of uterine infection this seed material affords luxuriant growths of *Br. abortus*, the organisms, indeed, may frequently be demonstrated in large numbers in suitably stained smears made from the tissues at the apposition of the fœtal and maternal discs. Following the abortion, or possibly the parturition, of an infected guinea-pig, single or multiple abscessation, with honeycombing and thickening of the uterine wall in the neighbourhood of the abscessation, frequently occurs. These lesions have been observed exclusively in *Br. abortus* infected animals, and in many cases the thickish cream-coloured pus from the abscesses yields abundant and pure cultures of *Br. abortus*, but at other times media inoculated with material from the uterine lesions is overgrown by contaminating bacteria.

Two groups of virgin female guinea-pigs were vaccinated, the one, Group (*a*), on March 21st, 1934; and the other, Group (*b*), on June 11th, 1934. The animals in both groups were each inoculated with 61,470 million organisms of Strain 45, those inoculated into Group (*a*) being from Sub-culture X15 and those used for the inoculation of Group (*b*) from Sub-culture X17. On June 11th a suitable number of male animals were introduced into each vaccinated group and also into a control group of normal females, Group (*c*). On July 2nd, 1934, all the female guinea-pigs received one drop on the eye of a saline suspension of a CO_2 sensitive strain of *Br. abortus* containing approximately 2,000 million bacteria per cubic centimetre. These animals were again infected in the same manner by applying a similar suspension to the opposite eye on July 5th. This double test dose of a virulent organism was very severe, experiments having demonstrated that very dilute suspensions of *Br. abortus*, when instilled on to the eye may set up infection with regularity. The animals

were killed from the twenty-eighth to the thirty-sixth day after first being infected, there being twelve females in Group (*a*), eleven in Group (*b*) and 21 in the control group (*c*). Unfortunately bacteriological examinations were made on only ten animals from Group (*a*), nine from Group (*b*) and 18 from the control group (*c*).

These bacteriological examinations showed that all the guinea-pigs were infected but the different groups were not affected to a like degree, the disease being mildest in Group (*a*) and most severe among the controls. Thus the average spleen weights were: Group (*a*) 1·72 grammes, Group (*b*) 2·27 grammes, and Group (*c*) 3·06 grammes, and the average estimated number of colonies per spleen was: Group (*a*) 9,600, Group (*b*) 61,800, and Group (*c*) 331,000. The superiority of the animals in Group (*a*) was maintained in the bacteriological examination of the livers and uteri. The examinations of the former organ showed none infected from Group (*a*); while four out of nine from Group (*b*) and 14 out of 18 in Group (*c*) were infected. The examinations of the uteri were particularly interesting and are summarised in Table V.

TABLE V.

Condition of Uterus.			*Cultures made from the Uterus or Placentae.*		
	Groups.			*Groups.*	
A.	*B.*	*C.*	*A.*	*B.*	*C.*
P.	P.	P.	———	None made	———
P.	P.	Abt.		" "	
		P.		" "	
P.	Abs.	P.	Neg.	Contin.	Contin.
P.	P.	P.	"	Neg.	"
P.		P.	Contin.		"
P.		P.	Neg.		"
		Not P.N.			Neg.
		"			"
P.	Abs.	"	Neg.	Contin.	"
		P.			Contin.
		P.			"
Abs.	Abs.	Abs.	O.B.	O.B.	O.B.
P.	Abs.	P.	Neg.	O.B.	Contin.
Not P.N.	P.	E.P.	"	Neg.	"
P.	P.	Abs.	"	Contin.	"
E.P.	P.	Abs.	"	"	"
	Not P.N.	Not P.N.		Neg.	Neg.
		E.P.			"
		Abs.			Contin.

P. = Pregnant; E.P. = Early pregnancy; Abt. = Abortion; Abs. = Abscessation of uterus; Not P.N. = Not pregnant, uterus normal.

Neg. = No bacterial growth; Contin. = Continuous growth of *Br. abortus* on surface of agar; O.B. = No growth of *Br. abortus* but a growth of other bacteria.

Considering those animals examined bacteriologically, it is seen that seven animals in Group (*a*) were well advanced in

pregnancy, and in only one of these was the uterus found infected. One animal in Group (*a*) showed abscessation of the uterus and one was in an early stage of pregnancy. *Br. abortus* was not isolated from the uterus of either of these animals but the abscessation is regarded as the direct sequel to *Br. abortus* infection. Therefore of the guinea-pigs which were pregnant or showed evidence of having been pregnant, only two out of nine, or 22 per cent., were infected or had suffered from a uterine infection with *Br. abortus*.

Seven control guinea-pigs were well advanced in pregnancy and all were infected in the uterus; four were suffering from abscessation of the uterus; two were in early pregnancy and one of these was infected. Therefore, out of 13 guinea-pigs which were pregnant or showed evidence of having been pregnant twelve, or 92 per cent., were, or had been, infected in the uterus with *Br. abortus*.

The animals in Group (*b*) were little, if at all, more resistant to uterine infection than the controls. The unsatisfactory results shown by Group (*b*) might be attributable to the use of a deteriorated vaccine. The only difference between the vaccines was that the vaccine inoculated into Group (*b*) animals was prepared from a culture two sub-cultures removed from that which provided the vaccine for Group (*a*), but it is unlikely that this was responsible for a deterioration of the vaccine.

The experiment in general confirmed the impression formed in 1931 that a considerable resistance to uterine infection could be induced by immunisation.

The next experiment was commenced in November 1934, its chief purpose being to obtain further information regarding the resistance of vaccinated guinea-pigs to *Br. abortus* infection acquired during pregnancy; both living and formalinised vaccines were tested.

Forty-eight female guinea-pigs believed to be non-pregnant were selected and divided into six groups. These animals were kept under observation for eight weeks prior to any inoculations being made and at the end of that period used for experiment. The groups were then treated as follows:—

Group 1.—Inoculated subcutaneously once with 6,148 million live organisms of Strain 45, Sub-culture X14.

Group 2 (six guinea-pigs).—Inoculated subcutaneously with 30 million live organisms, Strain 45, Sub-culture X14.

Group 3.—Inoculated subcutaneously with 30,740 million dead organisms, Strain 45, Sub-culture X14, formalinised vaccine.

Group 4.—Inoculated subcutaneously with 30 million dead organisms, Strain 45, Sub-culture X14, formalinised vaccine.

Group 5.—Inoculated subcutaneously with 30,740 million dead organisms of a virulent CO_2 sensitive strain No. 253, formalinised vaccine, prepared from Sub-culture 10.

Group 6.—Controls.

Four weeks after vaccination two male guinea-pigs were placed with each group of females and two weeks later the females each received a subcutaneous inoculation of what was estimated to be 30,700 live abortion bacteria of Strain 253, Sub-culture 12; the guinea-pigs were killed and examined 44 and 58 days later. The results of these examinations are shown in Table VI.

TABLE VI.

Group 1.	Live vaccine, moderate dose.	
	Titres of sera after vaccination and before infection :	1 : 80–1 : 320.
	No. of guinea-pigs examined, 8.	
	" " found pregnant, 6.	
	" " infected in the uterus, 0.	
Group 2.	Live vaccine, small dose.	
	Titres of sera after vaccination and before infection :	Negative at 1 : 10–1 : 160.
	No. of guinea-pigs examined, 6.	
	" " found pregnant, 1.	
	" " infected in the uterus, 1.	
Group 3.	Live vaccine strain sterilised by formalin.	
	Titres of sera after vaccination and before infection :	Negative at 1 : 10–1 : 40.
	No. of guinea-pigs examined, 8.	
	" " found pregnant, 1 (dead fœtus).	
	" " infected in the uterus, 6.	
Group 4.	Live vaccine strain sterilised by formalin, small dose.	
	Titre of sera after vaccination and before infection :	All negative at 1 : 10.
	No. of guinea-pigs examined, 8.	
	" " found pregnant, 0.	
	" " infected in the uterus, 6.	
Group 5.	Virulent strain sterilised by formalin.	
	Titres of sera after vaccination and before infection :	Negative at 1 : 10–1 : 40.
	No. of guinea-pigs examined, 8.	
	" " found pregnant, 0.	
	" " infected in the uterus, 5.	
Group 6.	Controls.	
	No. of guinea-pigs examined, 8.	
	" " found pregnant, 0.	
	" " infected in the uterus, 4.	

The control guinea-pigs, Group 6, were all heavily infected in the spleen and in all cases *Br. abortus* was recovered from the liver. None of the animals was pregnant at the time of the examination and in four cases macroscopic examination of the uterus showed no evidence of the animals having been pregnant. From the appearance of the uteri of the four remaining animals it is probable that they had aborted. *Br. abortus* was isolated in culture from the uteri of four of the eight guinea-pigs and the uteri of two of these four animals showed no macroscopic evidence of abortion having occurred. It is, however, probable that these two latter animals had aborted as *Br. abortus* is rarely recovered, by the technique used, from the uteri of infected non-pregnant guinea-pigs unless it be soon after abortion or from uteri showing

abscessation. The combined macroscopic and bacteriological evidence therefore indicates that six of the guinea-pigs had had infected uteri and probably had aborted.

In Group 1, vaccinated with a moderately large number of living micro-organisms of Strain 45, well-developed fœtuses were found in six of the guinea-pigs. With one of the two remaining animals it was very doubtful whether abortion had occurred, but in the other there was no evidence that the animal had been pregnant. In no case was *Br. abortus* isolated from the uterus or the placentæ, showing that the animals possessed a relatively high degree of resistance to a uterine infection. It has been found that *Br. abortus* is recoverable with much greater regularity from the placentæ of infected guinea-pigs than from the uterine mucosa of non-pregnant animals, which reflects more forcefully the immunity of the uterus in the six vaccinated pregnant animals. As a group, and as individuals, the infection of the spleen was much lighter than in the controls; indeed, two of the eight vaccinated animals were found free from infection altogether and in no instance was *Br. abortus* isolated from the liver tissue.

It is difficult to decide whether the resistance of the animals in Group 2 had been increased. Five of the animals were not pregnant and in three of these there was no macroscopic evidence of the uterus having been gravid, and *Br. abortus* was not recovered from the uteri; probably both of the others had aborted and *Br. abortus* was isolated from the uterus of one of them. The sixth guinea-pig was pregnant but its uterus was not infected. The spleens and livers showed a degree of infection comparable to that shown by the controls. The guinea-pigs do not compare favourably with those in Group 1, indicating that a single small inoculation of vaccine was relatively unsatisfactory.

In the remaining groups 3, 4 and 5 there was evidence that the majority of the animals had aborted and there was no evidence of any increased resistance to infection. It may therefore be concluded that the formalinised vaccines whether prepared from Strain 45 or from the virulent Strain 253, and this strain was used to test immunity, were of little value. Even when the formalinised vaccines were inoculated in very large quantity they were definitely very inferior to the vaccine composed of living non-virulent micro-organisms.

In conformity with what has been the general experience throughout all the experiments, the agglutination titres at the time the guinea-pigs were killed were highest among the control animals and those groups which showed no increased resistance to infection.

Attention should be directed to the pre-infection agglutination titres of the sera from some of the groups. The inoculation of very large numbers of killed micro-organisms of the virulent strain in no case produced a titre higher than 1 : 40. The inoculation of

similar numbers of killed micro-organisms of the non-virulent Strain 45 produced very similar titres, 1 : 40 again being the highest recorded. The inoculation of a relatively small number, 30 million, sterilised organisms of Strain 45 failed to produce agglutinins at all; however, 30 million of the live organisms of this strain caused the appearance of agglutinins in the majority of the animals, a titre of 1 : 160 being reached in one instance. Those animals in Group 1 inoculated with the largest number of living non-virulent organisms produced titres distinctly higher than the animals receiving five times as many killed organisms of the same strain or of the virulent strain. In all of the groups except No. 1 the titres showed a very marked rise after exposure to a small number of virulent micro-organisms. A general consideration of these results suggests : (1) That following sterilisation by formalin, Strain 45 stimulated the production of agglutinins as effectively as did the sterilised virulent strain; (2) that when the living organisms were inoculated the higher titres produced by the virulent strain depended upon the invasiveness of the strain and its multiplication and persistence for some considerable time in the tissues; (3) and that the relatively feeble production of agglutinins following the inoculation of living non-virulent micro-organisms was due to their inability to persist in the body tissues.

1935—*Experiments on Male Guinea-pigs.*

An experiment was carried out in which groups of guinea-pigs were vaccinated at different times and all infected at the same time. The chief object of the experiment was to test the length of time immunity lasted. Unfortunately no satisfactory immunity was demonstrated in any of the vaccinated groups although the methods of vaccination and of infecting the animals were regarded as comparable to those used in other experiments where a definitely increased resistance had been shown. By this time four years had elapsed since the earlier experiments had indicated that the inoculation of guinea-pigs with living organisms of Strain 45 stimulated an increased resistance to a subsequent *Br. abortus* infection. The poor results obtained in the last experiment suggested the possibility of a decided antigenic deterioration of the vaccine strain. Accordingly, arrangements were now made to compare the immunity of guinea-pigs inoculated with vaccine prepared from the ordinary sub-culture of Strain 45, with the immunity of guinea-pigs inoculated with vaccine prepared from a culture which had been passaged intermittently through a short series of guinea-pigs. In anticipation of an experiment of this nature the intermittent passage of Strain 45 had commenced in February 1935, and in June 1935 a sub-culture was available from Strain 45 which had been passaged through a series of six guinea-pigs and vaccine was prepared from it; the vaccine itself was obtained from the third sub-culture on liver agar.

For further comparative purposes a group of guinea-pigs was inoculated with a culture of Strain 45 which had been sub-cultivated 550 times since September 1931. Another group of guinea-pigs was inoculated with formalinised vaccine. Particulars of the vaccination of the groups of guinea-pigs and the test inoculation of virulent culture are as follows:—

Group 1.—Vaccinated with 6,148 million live organisms, Strain 45, Sub-culture X21.

Group 2.—Vaccinated with 6,148 million live organisms, Strain 45 passaged through six guinea-pigs, Sub-culture 3. The culture from which the vaccine was prepared consisted of rather a sparse growth, the media apparently not being up to the usual standard of quality.

Group 3.—The vaccine was in all respects comparable to that used for Group 2, except that the vaccine was prepared from a culture of very good growth.

Group 4.—Vaccinated with 6,148 million live organisms of Strain 45, Sub-culture X550.

Group 5.—Vaccinated with 6,148 million organisms of Strain 45, Sub-culture X21, sterilised by the action of 0·25 per cent. formalin acting at 37° C. for 48 hours.

Group 6.—Controls.

A test inoculation of 300 organisms was given 35 days after the inoculation of the single dose of vaccine. The strain was the same as that used in the previous experiment but the numbers of organisms used was 100 times fewer. The reduction was made because of the possibility that the test inoculation in the preceding experiment had been too severe.

The guinea-pigs were examined in equal numbers from each group at from 38 to 47 days after receiving the test inoculation. The results of the experiment are summarised in Table VII.

Accepting the absence of infection from all the organs examined as the most reliable criterion for assessing the resistance of the groups, it is evident that both Group 2 and 3, which had been vaccinated with the animal-passaged strain, were the most resistant, a comparison of these two groups with the control group showing a difference of 80 per cent. in favour of the former. The two groups 1 and 4 inoculated with vaccine prepared from Sub-culture X21 and Sub-culture X550 respectively showed a 50 per cent. greater resistance to infection than the controls, which probably reflects a definite degree of immunity produced by the vaccination. Group 5 inoculated with formalinised vaccine showed a superiority of only 20 per cent. over the controls.

Infection of the liver and kidney not being detected regularly among the controls, furnish records of limited value, as do the spleen weights. But upon whichever of these data the groups be compared, Nos. 2 and 3 are found to be the most resistant. An intermediate position is occupied by Groups 4 and 5, except that

TABLE VII.

Group.	*No. of G. Pigs Examined.*	*No. found Infected.*	*No Heavily Infected in Spleen.*	*No. Infected in Liver.*	*No. Infected in Kidney.*	*Average Weight of Spleens.*	*Remarks.*
1. Vaccinated. Str. 45, Sub. X21	10	4	1	2	1	1·32 grammes.	Two showed macroscopic lesions of the spleen.
2. Vaccinated. Passaged Str. 45	10	1	1	0	0	1·13 grammes.	None showed lesions.
3. Vaccinated. Passaged Str. 45	10	1	0	0	0	1·00 gramme.	None showed lesions.
4. Vaccinated. Str. 45, Sub. X550	10	4	1	0	1	1·43 grammes.	Two showed macroscopic lesions of the spleen.
5. Vaccinated. Str. 45, Sub. X21 Formalinised	10	7	3	3	0	2·39 grammes.	Four showed macroscopic lesions of the spleen.
6. Controls	10	9	4	1	2	1·95 grammes.	Seven showed macroscopic lesions of the spleen and three showed macroscopic lesions of one or both epididymi.

Even after animal passage the " ærobic " vaccine strain was not recovered from the guinea-pigs in Groups 2 and 3.

in the case of Group 5 the spleen weights being heavier than those shown by the controls suggests that in this particular respect Group 5 were no more resistant than the control animals and this therefore relegates Group 5 to a position subordinate to both Groups 1 and 4; hence the relative position of the three groups is again found to be the same as when resistance was assessed on the numbers of animals in each group found to be free from infection. The superior resistance of animals in Groups 2 and 3 suggests that animal passage had improved Strain 45 as a vaccine, without resuscitating virulence for these animals.

The test inoculation in this experiment was sufficiently severe to cause infection in nine out of ten control animals, but not too drastic to break through the resistance of all members in the vaccinated groups.

Summary.

The immunisation of guinea-pigs inoculated with living micro-organisms of a non-virulent strain of *Br. abortus* is recorded. The strain though smooth and fully agglutinable does not stimulate the production of agglutinins in high titre even when inoculated in very large amounts. This is attributed to the lack of invasiveness and the inability of the organisms to persist in the body of the inoculated animal and not to defective or deficient antigenic structure.

Female guinea-pigs when pregnant were found highly susceptible to a uterine infection with *Br. abortus*, death of the fœtus or fœtuses and abortion frequently occurring. After abortion, abscessation of the uterus was sometimes encountered and it is regarded as specific evidence of a uterine infection with *Br. abortus*. Female guinea-pigs vaccinated with the non-virulent strain before pregnancy and infected whilst pregnant or when running with male animals, though infected in the spleen, were shown to possess a high degree of resistance to a uterine infection which was not enjoyed by control animals. Male guinea-pigs inoculated with either single or multiple large doses of the non-virulent living organisms showed in some experiments a markedly increased resistance to infection, enabling the results of the experiments to be judged by a comparison of the number of infected vaccinated animals with the number of infected control animals, to the decided advantage of the former. Evidence was obtained suggesting that vaccinated animals, though not in all cases completely resistant to an infection, showed very considerable resistance to a second or superimposed infection. This does not, however, indicate that immunity necessarily depends upon the persistence of infection. Guinea-pigs inoculated with the non-virulent strain were found to be resistant after a period of 19 weeks, the longest period tested. As there is evidence to show that the non-virulent strain rarely persists in the body of an inoculated

guinea-pig for more than three weeks, an immunity lasting for 19 weeks may be regarded as one which is independent of the persistence of infection in the body.

Comparative tests of vaccine prepared from different sub-cultures of the non-pathogenic strain, which were separated by over 200 sub-cultivations in one instance and by more than 500 in the other, do not indicate any definite antigenic deterioration caused by repeated sub-cultivation alone.

The non-virulent strain after " intermittent " passage through a series of six guinea-pigs was still non-pathogenic, but there was some evidence that as a vaccine it was superior to the non-passaged strain. The evidence is too limited to warrant any conclusion, but it indicates one direction in which further experiments should be directed.

Although in the majority of experiments clear-cut results were obtained this was not always the case. Why in a few instances vaccinated groups of guinea-pigs showed little or no more resistance than the control animals is still obscure.

The killed vaccines tested were unsatisfactory.

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The Influence of Preparturient Intramammary Vaccination on Immunoglobulin Levels in Bovine Mammary Secretions

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Summary. Immunoglobulin concentrations were measured in colostrum and milk from individual mammary glands of three cows. Two of the glands of each cow had been vaccinated with a live, formalinized, *E. coli* vaccine. No major differences were found between immunoglobulin concentrations in colostrum from vaccinated and non-vaccinated glands. However, markedly higher concentrations of all immunoglobulins were found in mammary secretions from the vaccinated glands taken 2 and 3 days after calving. IgA concentrations were significantly higher in milk from vaccinated glands than in milk from non-vaccinated glands from day 2 to day 28.

INTRODUCTION

The first suggestion that the mammary gland could be stimulated to produce antibodies was made by Smith, Orcutt and Little (1923). This was based on the observation that cows with active Brucella infections of the mammary gland, and cows vaccinated in the mammary gland with a killed Brucella vaccine, had higher antibody titres in the milk than did cows vaccinated parenterally or cows with non-infected glands.

Mitchell, Walker and Bannister (1953; 1954) recorded the secretion of high levels of neutralizing antibodies to viruses which had been instilled into the mammary glands of cattle. Antibodies were demonstrated later in milk from the non-vaccinated glands and blood serum than in milk from the vaccinated gland. Wilson (1972) demonstrated that cows vaccinated in the mammary gland with *Escherichia coli* antigens responded by secreting milk which contained significantly higher antibody titres than did milk from the non-vaccinated glands.

Enhanced antibody secretion from the mammary gland following local vaccination has been shown in goats (Mitchell, Guerin and Robillard, 1969; Pasieka, Guerin and Mitchell, 1970), sheep (Lascelles, Outteridge and Mackenzie, 1966; McDowell and Lascelles, 1969) and pigs (Wilson, Svendsen and Brown, 1972).

The ability of the bovine and ovine mammary gland selectively to concentrate IgG1 from serum immediately prior to parturition has been recognized (Murphy, Aulund, Osebold and Carroll, 1964; Pierce and Feinstein, 1965; Aalund, 1968). Even though

the absolute immunoglobulin levels are much lower, the electrophoretically fast IgG continues to be selectively transported during lactation in sheep (Mackenzie and Lascelles, 1968) and cattle (Dixon, Weigle and Vasquez, 1961).

The concentration of immunoglobulins in bovine milk and colostrum have been measured by Mach and Pahud (1970), Klaus, Bennett and Jones (1969) and Butler (1971). The purpose of this study was to measure the concentration of the immunoglobulins in bovine colostrum and milk from vaccinated and non-vaccinated mammary glands.

MATERIALS AND METHODS

Cows

Three 18-month-old heifers of the Jersey breed were used. At the start of the experiment it was estimated that they were approximately 1 month from calving.

Vaccine

A tryptic soy broth culture containing $7–8 \times 10^8$ viable bacteria/ml was incubated for 15 hours at 37° in the presence of 0·04 per cent v/v of formalin. The formalinized cultures contained approximately 7×10^6 viable bacteria/ml.

Vaccination

Vaccine was introduced into the teat cistern of the right front (RF) and left hind LH mammary gland via the teat canal.

Heifer 2Z was vaccinated with an antigen prepared from *E. coli* serotype 08: K87; 88a, b; H19. (P307 strain): 4 ml and 6 ml were inoculated into each gland 15 and 5 days before calving respectively.

Heifer W3 was vaccinated with an antigen prepared from *E. coli* serotype 0149:K91; 88a, c; H10 (A1 strain): 4, 6 and 8 ml were inoculated into each gland, 26, 16 and 6 days before calving respectively.

Heifer 3Z was vaccinated with 4, 6 and 8 ml into each gland 28, 18, 8 and 1 days before calving, respectively. The RF gland was inoculated with P307 and the LH gland with A1 vaccines.

Whey

Colostrum or milk was taken from each mammary gland on the day of parturition and on days 2, 3, 7, 14 and 28 post-parturition. These samples are referred to as d1, d2, d3, d7, d14 and d28, respectively.

Whey was prepared from the samples by centrifugation at 44,000 *g* for 2 hours (Bohren and Wenner, 1961) and stored in 2-ml aliquots at −30°.

Antisera

Antisera used in this study were produced in guinea-pigs following the techniques of Binaghi, Orisol and Boussac-Aron (1967). The antigen consisted of immunoprecipitates of the respective immunoglobulins obtained by immunoelectrophoresis (precipitated with guinea-pig antisera having class specificity) and incorporated into Freund's complete adjuvant. Anti-IgA and anti-IgM antisera did not require absorption to render them monospecific as measured by micro gel diffusion, immunoelectrophoresis and radial

immunodiffusion using purified standards of other immunoglobulin classes and a semi-purified secretory component preparation. Most anti-IgG1 and anti-IgG2 antisera failed to react with IgA and IgM but did cross react with the other IgG subclasses. Absorption of these anti-IgG subclass antisera with the other IgG subclasses rendered them monospecific. (Duncan, Wilkie, Hiestand and Winter, 1972).

Measurement of immunoglobulins

Immunoglobulins were measured in serum and secretions by single radial immunodiffusion by the method of Fahey and McKelvey (1964) with some modifications. Plates containing appropriate amounts of antisera (1·5 per cent agar in 0·1 M Tris-HCl buffer pH 7·4) were allowed to diffuse for 24 hours at room temperature for measurement of the IgG1, IgG2 and IgA. IgM plates were allowed to diffuse for 48 hours. Plates were stained with 5 per cent acetic acid (Kaufman, 1970), and the ring diameter measured on a microcomparator (Nikon Profile projector). Six standards were included on each plate using purified preparations of IgG1 (colostrum; 6·8S), IgG2 (serum; 6·7S), and IgA secretory; predominately 11S) suspended on 0·1 M Tris-HCl buffer, pH 7·4, containing 1 mM disodium ethylene diamine-tetraacetate (EDTA). Dilutions of bovine serum for which IgM concentrations had been determined by comparison to known concentrations of the purified IgM, were used for IgM standards. The preparation of the immunoelectrophoretic standards has been described by Duncan *et al.* (1972).

The reproducibility of the tests performed is demonstrated by the range and mean coefficients of variation on the six dilutions of immunoglobulin standards from tests performed on the same day; IgG2, 0·10–3·8 per cent ($\bar{X}$ = 1·38); IgG1, 0·34–5·4 per cent ($\bar{X}$ = 2·3); IgA, 0·12–3·8 per cent ($\bar{X}$ = 1·2); IgM 0·60–5·7 per cent ($\bar{X}$ = 2·6).

Statistical analysis

The paired *t*-test (Alder and Roessler, 1967) was used to compare immunoglobulin concentration in whey from vaccinated and non-vaccinated glands. Differences with calculated *t* values greater than at the 0·05 level of probability were considered significant.

RESULTS

The concentration of IgG1, IgG2, IgM and IgA in mammary secretions on days 1 to 28 after calving from immunized and non-immunized mammary glands are given in Table 1. The mean immunoglobulin levels in vaccinated and non-vaccinated gland secretions are shown in Table 2.

IgG1 was found to be the predominant immunoglobulin in colostrum, the levels dropping rapidly to day 7 and more slowly thereafter. Apart from the day 1 sample the mean IgG1 concentration was consistently higher in milk from vaccinated glands, especially on days 2 and 3, statistically significant differences were seen between IgG1 levels on days 2, 3, 7 and 28. IgG1 was still the predominant immunoglobulin in secretions taken 28 days after calving.

IgG2 was from 1/6 to 1/20 the level of IgG1 in samples taken on day 1 and, like IgG1, decreased rapidly to day 7 and more slowly thereafter. Significant differences in IgG2 concentrations between vaccinated and non-vaccinated secretions were found on days 2, 3 and 28.

TABLE 1

IMMUNOGLOBULIN CONCENTRATIONS IN MAMMARY SECRETIONS FROM COWS VACCINATED IN THE MAMMARY GLAND WITH *Escherichia coli* ANTIGENS

Cow No./Ig	Gland	Days after calving					
		1	2	3	7	14	28
		(mg/ml)					
2Z/IgM	RH	3·15	0·54	0·11	0·15	0·125	0·03
	RF	3·35	3·40	0·68	0·10	0·105	0·03
	LH	3·2	0·76	0·38	0·14	0·14	0·03
	LF	3·65	0·28	0·125	0·21	0·17	0·04
2Z/IgG1	RH	41·0	8·0	1·89	0·60	0·34	0·24
	RF	35·0	18·0	2·60	0·72	0·44	0·31
	LH	36·0	13·5	2·44	0·64	0·37	0·275
	LF	38·0	3·85	1·71	0·62	0·34	0·22
2Z/IgG2	RH	4·0	0·67	0·18	0·08	0·04	0·03
	RF	4·25	2·25	0·82	0·08	0·055	0·04
	LH	3·70	1·60	0·44	0·10	0·05	0·04
	LF	3·80	0·44	0·19	0·06	0·05	0·03
2Z/IgA	RH	1·35	0·255	0·09	0·08	0·09	0·07
	RF	1·47	1·04	0·32	0·22	0·28	0·22
	LH	1·45	0·81	0·28	0·18	0·24	0·16
	LF	1·65	0·123	0·07	0·07	0·10	0·09
W3/IgM	RH	2·53	0·60	0·25	0·18	0·11	0·08
	RF	2·33	1·26	0·51	0·22	0·08	0·12
	LH	3·06	0·87	0·40	0·26	0·15	0·11
	LF	3·46	0·50	0·32	0·26	0·11	0·10
W3/IgG1	RH	17·7	3·75	1·42	0·50	0·34	0·23
	RF	12·1	4·85	2·23	0·60	0·26	0·29
	LH	21·0	5·50	1·83	0·68	0·41	0·33
	LF	24·5	3·95	1·26	0·60	0·36	0·30
W3/IgG2	RH	0·95	0·22	0·09	0·07	0·04	0·03
	RF	0·86	0·49	0·17	0·07	0·04	0·04
	LH	0·91	0·54	0·15	0·09	0·06	0·05
	LF	1·03	0·28	0·11	0·07	0·05	0·04
W3/IgA	RH	0·98	0·12	0·07	0·05	0	0·05
	RF	1·06	0·68	0·325	0·16	0·12	0·09
	LH	1·02	0·40	0·21	0·15	0·10	0·065
	LF	0·59	0·11	0·06	0·05	0·05	0·05
3Z/IgM	RH	6·1	2·0	0·29	0·13	0·145	0·07
	RF	4·4	3·10	0·46	0·12	0·09	0·05
	LH	4·4	3·70	1·45	0·12	0·08	0·05
	LF	4·2	1·15	0·22	0·13	0·105	0·06
3Z/IgG1	RH	49·0	27·0	2·34	0·85	0·32	0·37
	RF	33·0	33·0	5·7	0·96	0·305	0·43
	LH	25·5	32·5	8·6	1·17	0·77	0·5
	LF	50·0	20·0	2·95	0·80	0·35	0·375
3Z/IgG2	RH	5·75	1·45	0·39	0·09	0·05	0·03
	RF	6·0	3·8	0·76	0·11	0·05	0·05
	LH	6·3	3·4	2·8	0·16	0·09	0·05
	LF	5·5	1·4	0·28	0·08	0·055	0·04
3Z/IgA	RH	2·25	0·43	0·152	0·10	0·08	0·07
	RF	3·5	1·15	0·47	0·195	0·165	0·14
	LH	1·7	1·53	1·27	0·355	0·36	0·27
	LF	1·94	0·31	0·17	0·08	0·06	0·07

Cow 2Z vaccinated in RF and LH with *E. coli* serotype 08 : K87; 88a, b: H19 (P307 strain).
Cow W3 vaccinated in RF and LH glands with *E. coli* serotype 0149 : K91; 88a, c: H10 (A1 strain).
Cow 3Z vaccinated in RF gland with the A1 strain and in the LH gland with the P307 strain of *E. coli*.
RH = right hind; RF = right fore; LH = left hind; LF = left fore.

TABLE 2

MEAN IMMUNOGLOBULIN CONCENTRATIONS IN SECRETIONS FROM VACCINATED AND NON-VACCINATED MAMMARY GLANDS OF COWS

Ig	Treatment	Days after calving					
		1	2	3	7	14	28
		(mg/ml)					
IgM	Non-vaccinated	3·85 (1·12)	0·85 (0·56)	0·22 (0·07)	0·18 (0·05)	0·18 (0·0)	0·06 (0·0)
	Vaccinated	3·46 (0·74)	2·18* (1·24)	0·65*(0·37)	0·16 (0·06)	0·11 (0·0)	0·07 (0·0)
IgG1	Non-vaccinated	34·04 (11·37)	11·09 (9·12)	1·93 (0·57)	0·66 (0·12)	0·34 (0·0)	0·29 (0·05)
	Vaccinated	27·10 (8·57)	17·89*(11·43)	3·90*(2·46)	0·79*(0·20)	0·43 (0·16)	0·36* (0·08)
IgG2	Non-vaccinated	3·50 (1·91)	0·74 (0·50)	0·21 (0·10)	0·07 (0·0)	0·05 (0·0)	0·03 (0·0)
	Vaccinated	3·67 (2·18)	2·01 (1·28)	0·86*(0·90)	0·10 (0·0)	0·06 (0·0)	0·045*(0·0)
IgA	Non-vaccinated	1·46 (0·56)	0·22 (0·13)	0·10 (0·04)	0·07 (0·14)	0·06 (0·03)	0·06 (0·01)
	Vaccinated	1·67 (0·77)	0·94* (0·36)	0·48*(0·36)	0·21*(0·68)	0·21*(0·09)	0·16 *(0·07)

Vaccinated cows received *E. coli* antigens in the right front and left hind mammary glands. Figures in parentheses are standard deviations of the mean.

* Significant difference between mean of vaccinated and non-vaccinated.

IgM concentrations were approximately equal to IgG2 levels in cows 2Z and 3Z but in cow W3 were three times the IgG2 levels. As for IgG1 and IgG2, the difference in IgM levels between vaccinated gland secretions were particularly marked on days 2 and 3. The differences on days 2 and 3 were statistically significant.

Day 1 IgA concentrations were lower than any other immunoglobulin. The IgA concentration in secretions from vaccinated glands on days 2, 3, 7, 14 and 28, were significantly greater than the concentrations found in milk from non-vaccinated glands. Absolute levels of IgA in secretions from both vaccinated and non-vaccinated glands were higher than IgG2 in all cows on days 14 and 28 and higher than IgM in cows 2Z and 3Z on day 28.

The ratio of IgG1 and IgG2 in day 1 secretions was approximately 10:1 and was the same in day 28 secretions.

The marked difference in immunoglobulin concentration in the colostral (day 1) samples among cows and among quarters in the same cow (independent of vaccination) should be noted.

DISCUSSION

Preparturient intramammary vaccination has clearly altered the immunoglobulin content of post-parturient bovine mammary secretions. The milk whey from vaccinated glands contained greater concentrations of IgG (IgG1 and IgG2) and IgM on days 2 and 3 and IgA on days 2 to 28. Mechanisms responsible for this could be an increase in vascular permeability, an increase in selective transport from serum, or production of the immunoglobulins in the mammary gland.

Mechanisms responsible for the large differences in immunoglobulin concentrations between samples taken from vaccinated and non-vaccinated glands on days 2 and 3 remain obscure. Since IgM and IgG2 concentrations did not differ so markedly in secretions from vaccinated and non-vaccinated glands taken after day 3 it would suggest that the initial increase was due to either non-selective transport from serum, or enhanced local antibody synthesis of IgM, and IgG2 which was of a much shorter duration than that of IgA. The significant increases in IgA and IgG1 levels associated with vaccination are probably the result of local antibody production in the mammary gland to either the broth diluent or the bacterial antigens. Increased levels of IgA (Lascelles and McDowell, 1970), and greater numbers of IgA synthesizing plasma cells (Lee and Lascelles, 1970), have been demonstrated in antigenically stimulated mammary glands from ewes.

In this study the use of 11S IgA standards to quantify secretory IgA immunoglobulins would tend to give lower readings than may exist if IgA immunoglobulins of a higher sedimentation velocity were present. Monomeric (7S) IgA has not been demonstrated in cattle. (Mach and Pahud, 1970; Vaerman, 1970; Duncan *et al.*, 1972).

Bovine IgG1 is preferentially secreted into colostrum prior to parturition (Murphy *et al.*, 1964; Pierce and Feinstein, 1965; Dixon *et al.*, 1961). The ratio of IgG1:IgG2 in colostrum found in this study was approximately 10:1 and the same ratio existed in day 28 milk. Therefore, if a selective transfer of IgG1 operates on day 1, the same mechanism would appear to operate on day 28. In contrast, an IgG1:IgG2 ratio of 3:1 to 2:1 has been found in nasal secretions and tears from non-lactating heifers (Duncan *et al.*, 1972). It remains to be seen what influence lactation has on IgG transport to other epithelial membranes.

Although marked increases were not found in the concentration of any of the immunoglobulin classes between vaccinated and non-vaccinated gland samples taken on day 1, qualitative differences were found in the form of increased indirect haemagglutinin antibody titres in the samples from vaccinated glands. Bactericidal activity was not found in samples taken from any gland on any day, but inhibition of bacterial multiplication occurred in all samples through day 3 and was demonstrable in vaccinated gland samples to day 28. (Wilson, 1972). Studies to determine in which immunoglobulin class the specific antibody activities reside have not been performed.

The enhanced immunoglobulin production in the vaccinated mammary gland which persisted for up to 28 days after antigen administration could be of practical significance in the prevention of mastitis, or in prophylaxis against enteric infections in newborn animals ingesting milk secreted from such glands. Since secretory immunoglobulin is more resistant to digestion than other immunoglobulins (Brown, Newcomb and Ishizaka, 1970; Stewart, 1971) and has the tendency to adhere to epithelium and resist absorption South, 1971), the increased levels of immunoglobulin found in milk as a result of local vaccination (and presumably specific antibody in the same class) could be of major significance as far as immune prophylaxis to enteric infections is concerned.

The marked inter-cow variation of absolute immunoglobulin concentrations in day 1 mammary secretions could be of importance in the pathogenesis of hypogammaglobulinaemia of newborn calves since the absolute mass of immunoglobulin ingested (Kruse, 1970) does influence the subsequent serum immunoglobulin levels attained by calves.

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Attenuation and Vaccine Potential of *aroQ* Mutants of *Corynebacterium pseudotuberculosis*

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Corynebacterium pseudotuberculosis*, a gram-positive intracellular bacterial pathogen, is the etiological agent of the disease caseous lymphadenitis (CLA) in both sheep and goats. Attenuated mutants of *C. pseudotuberculosis* have the potential to act as novel live veterinary vaccine vectors. We have cloned and sequenced the *aroB* and *aroQ* genes from *C. pseudotuberculosis* C231. By allelic exchange, *aroQ* mutants of both C231, designated CS100, and a *pld* mutant strain TB521, designated CS200, were constructed. Infection of BALB/c mice indicated that introduction of the *aroQ* mutation into C231 and TB521 attenuated both strains. In sublethally infected BALB/c mice, both CS100 and CS200 were cleared from spleens and livers by day 8 postinfection. The in vivo persistence of these strains was increased when the intact *aroQ* gene was supplied on a plasmid in *trans*. Mice infected with TB521 harbored bacteria in organs at least till day 8 postinfection without ill effect. When used as a vaccine, only the maximum tolerated dose of CS100 had the capacity to protect mice from homologous challenge. Vaccination with TB521 also elicited protective immunity, and this was associated with gamma interferon (IFN-γ) production from splenocytes stimulated 7 days postvaccination. The role of IFN-γ in controlling primary infections with *C. pseudotuberculosis* was examined in mice deficient for the IFN-γ receptor (IFN-γR$^{-/-}$ mice). IFN-γR$^{-/-}$ mice cleared an infection with CS100 but were significantly more susceptible than control littermates to infection with C231 or TB521. These studies support an important role for IFN-γ in control of primary *C. pseudotuberculosis* infections and indicate that *aroQ* mutants remain attenuated even in immunocompromised animals. This is the first report of an *aroQ* mutant of a bacterial pathogen, and the results may have implications for the construction of aromatic mutants of *Mycobacterium tuberculosis* for use as vaccines.

Corynebacterium pseudotuberculosis, a gram-positive facultative intracellular pathogen, is the etiological agent of caseous lymphadenitis (CLA) in sheep and goats. CLA is a chronic disease typically characterized by necrotizing inflammation of one or more superficial lymph nodes. In sheep, CLA results in reduced wool production and meat losses due to carcass condemnation (31). In Australia, it is one of the most prevalent diseases of sheep, with economic losses in the order of $20 million per year (30). While the pathogenic process employed by *C. pseudotuberculosis* in causing CLA in sheep and goats is not well defined, at least two major virulence determinants have been identified. One of these is the toxic lipid cell wall, which may mediate the bacterium's resistance to killing by phagocytic cells (10). The other identified virulence determinant is a sphingomyelin-degrading phospholipase D (Pld) exotoxin (22). Pld is thought to mediate dissemination of the pathogen within the host by increasing local vascular permeability (3). A role for Pld in the virulence of *C. pseudotuberculosis* was confirmed when an isogenic *pld* mutant was constructed and shown to be unable to cause CLA. Importantly, sheep immunized with a *pld* mutant were protected from subsequent challenge with the wild-type parental strain (13). This Δ*pld* mutant holds promise as a veterinary vaccine vector, since it is capable of eliciting immune responses to coexpressed antigens in vaccinated sheep (14). There is, however, accumulating evidence to suggest that the type of mutation used to attenuate a vaccine vector can have a critical influence on the vector's ability to elicit an immune response to a carried foreign antigen (23). This most probably reflects the altered in vivo growth rate or persistence of the pathogen and coincident altered interaction with the immune system.

Several different bacterial pathogens have been attenuated by stable introduction of mutations in the aromatic amino acid biosynthetic pathway. Aromatic-dependent mutants of the following pathogens have been shown to be attenuated and capable of stimulating protective immunity in different animal models: *Salmonella typhimurium* (17), *Salmonella typhi* (26), *Salmonella cholerasuis* (27) *Shigella flexneri* (43), *Bordetella pertussis* (36), *Pasteurella multocida* (18), *Bacillus anthracis* (20), *Aeromonas salmonicida* (42), *Yersinia enterocolitica* (4), and *Yersinia pestis* (29). The reduced virulence of these bacterial strains is likely to be due to their requirement for *p*-aminobenzoic acid, a precursor of folic acid and a compound which is not synthesized by chordates. Since bacteria are unable to take up exogenous folate and the availability of *p*-aminobenzoic acid is limited in vertebrate tissues, the growth of *aro* mutants in vivo is severely restricted.

Here we report the cloning of the *aroB* and *aroQ* genes from *C. pseudotuberculosis* and their similarity with the corresponding genes from *Mycobacterium tuberculosis*. An *aroQ* mutant of *C. pseudotuberculosis* was constructed by allelic exchange, and experiments were conducted to test its efficacy as a vaccine in a murine model of infection. We believe this to be the first rationally attenuated prechorismate *aro* mutant of a gram-positive bacterium. The construction of an aromatic mutant of

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TABLE 1. Bacterial strains and plasmids used in this present study

Strain or plasmid	Relevant characteristics	Reference or source
C. pseudotuberculosis		
C231	Wild type	13
TB521	*pld* mutant of C231; generated by in vitro site-directed mutagenesis of *pld* sequence encoding Pld active site (His20→Ser20), then allelic exchange with wild-type gene in C231	41 and unpublished data
CS100	C231 *aroQ*::*erm*	This study
CS200	TB521 *aroQ*::*erm*	This study
E. coli		
AB2829	K-12 *aroA* mutant	A. J. Pittard (32)
AB2826	K-12 *aroB* mutant	A. J. Pittard (32)
AB2830	K-12 *aroC* mutant	A. J. Pittard (32)
AB1360	K-12 *aroD* mutant	A. J. Pittard (32)
BRD728	Lambda lysogen	Gift from G. Dougan
JM101	Cloning host	
DH5α	Cloning host	
Plasmids		
pHC79	Cosmid cloning vector	16
pBluescript	Cloning vector	Stratagene, La Jolla, Calif.
pEP-2	*E. coli-C. pseudotuberculosis* shuttle vector	34
pUC4K	Source of *Kan* cassette	Pharmacia, Piscataway, N.J.
pBTB24	Source of *erm* cassette	15
pCS1	*aroB*/*aroD* complementing cosmid	This study
pCS2	3-kb *Bam*HI fragment from pCS1 containing *aroB* and *aroQ* genes	This study
pCS3	3-kb *Bam*HI fragment from pCS2 blunt-end ligated to *Pvu*II-digested pBluescript	This study
pCS4	*kan* gene blunt-end ligated to *Sca*I-digested pCS3	This study
pCS5	*kan* gene blunt-end ligated to *Sca*I-digested pBluescript	This study
pCS6	*erm* gene blunt-end ligated to *Eco*RI-digested pCS4	This study
pCS7	*erm* gene blunt-end ligated to *Pvu*II-digested pCS5	This study
pCS8	3-kb *Bam*HI fragment from pCS2 in pEP-2	This study

C. pseudotuberculosis will facilitate an immunobiological comparison with the previously constructed Δ*pld* mutant with respect to efficacy as a CLA vaccine and also as a vaccine vector.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The properties of the bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were routinely grown in Luria-Bertani (LB) broth or agar supplemented with ampicillin (50 μg/ml), kanamycin (50 μg/ml), or erythromycin (200 μg/ml) when appropriate. M9 minimal medium (37) containing ampicillin and essential nonaromatic amino acids was used to select for cosmid clones which complemented the *E. coli* aromatic mutants. *C. pseudotuberculosis* strains were cultured in brain heart infusion (BHI); (Oxoid, Basingstoke, Hampshire, England) broth or agar for 2 days at 37°C. When appropriate, the medium was supplemented with erythromycin (150 ng/ml) or kanamycin (50 μg/ml).

DNA manipulation and analyses. Plasmid DNA preparation, restriction enzyme digests, DNA hybridization, ligations, and transformations were performed by using standard techniques (37). DNA fragments were purified from agarose gels by using Geneclean (Bio 101 Inc., Vista, Calif.). DNA probes were prepared by nick translation using [α-^{32}P]dATP (Amersham International, Buckinghamshire, England) according to the manufacturer's instructions. Similarly, DNA probes were stripped from Hybond-N (Amersham) membranes according to the manufacturer's instructions. DNA sequencing was performed by the dideoxy-chain termination method, using fluorescein-labeled dideoxynucleotides. The DNA sequence was analyzed with an Applied Biosystems 373A DNA sequencer (Perkin-Elmer, Melbourne, Victoria, Australia). Specific synthetic oligonucleotides and commercial (Promega Corp., Madison, Wis.) universal and reverse oligonucleotides were used in sequencing. C231 genomic DNA for construction of the cosmid library was a kind gift from Catherine Pogson (CSIRO Division of Animal Health). Genomic DNA from *C. pseudotuberculosis* was isolated by the whole-cell lysate technique (44).

Construction of a cosmid genomic library. C231 genomic DNA was partially digested with *Sau*3A and fractionated on a 10 to 40% sucrose gradient. DNA fragments in the 35- to 50-kb range were ligated to the cosmid vector pHC79, digested with *Bam*HI, and dephosphorylated with calf intestinal alkaline phosphatase. Aliquots of the ligation were packaged in vitro into bacteriophage lambda, using a commercial packaging kit (Boehringer GmbH, Mannheim, Germany).

Packaged cosmid clones were transduced into *E. coli* BRD728, which harbors a defective bacteriophage lambda lysogen stably maintained at 30°C. Cells were prepared for transduction according to the method supplied with the packaging kit. The library was amplified essentially as described by Jacobs et al. (21) and stored under chloroform at 4°C.

Complementation analysis. Repackaged recombinant cosmid molecules were transduced into cells of the particular *E. coli* aromatic auxotroph, prepared as described above for bacteriophage lambda infection, at a multiplicity of infection of 0.1. After a 30-min absorption period, 1 ml of LB broth was added and the cells were incubated at 37°C for 45 min to allow expression of antibiotic resistance genes. Cells were then washed twice with saline before being plated onto M9 minimal medium containing ampicillin.

Construction of aromatic mutants by allelic exchange. A suicide plasmid construct which could mediate allelic exchange and the generation of an *aroQ* mutant of *C. pseudotuberculosis* was constructed. The 3-kb *Bam*HI fragment in pCS2 was excised with *Bam*HI, made blunt-ended by using the Klenow fragment of DNA polymerase, and ligated to *Pvu*II-digested pBluescript KS$^+$ to produce pCS3. The *Eco*RI site 324 bp downstream from the putative ATG of the *aroQ* open reading frame was now unique in pCS3. The unique *Sca*I site located in the ampicillin resistance gene in pBluescript was then used. A *Hin*cII fragment containing the kanamycin resistance cassette from pUC4K was blunt-end ligated to *Sca*I-digested pCS3 and also to *Sca*I-digested pBluescript, generating pCS4 and pCS5, respectively. Finally, the *aroQ* gene was insertionally inactivated by blunt-end ligation of an erythromycin resistance cassette from pBTB24 to the blunt-ended (Klenow fragment) unique *Eco*RI site within the *aroQ* open reading frame in pCS4 to generate pCS6. The same erythromycin resistance cassette was also ligated to *Pvu*II-digested pCS5 to generate pCS7. Thus, pCS7 is identical to pCS6 but lacks the 3-kb *Bam*HI fragment harboring the *aroB* and -*Q* genes. pCS7 was used as a control to screen for the frequency of illegitimate recombination of plasmid sequences with the *C. pseudotuberculosis* chromosome. *C. pseudotuberculosis* C231 and pTB521 were electroporated with 5 μg of the appropriate plasmid construct (pCS6 or pCS7). Following electroporation, putative *aroQ* mutants were selected on BHI plates containing 150 ng of erythromycin per ml for 4 days at 37°C. Erythromycin-resistant colonies were presumed to result from a recombination event. Bacteria which had undergone an allelic exchange event whereby the entire plasmid had been integrated (merodiploid) would also be kanamycin resistant. Erythromycin-resistant colonies were patched onto BHI agar containing kanamycin. Erythromycin-resistant, kanamycin-sensitive colonies were subsequently analyzed by Southern hybridization for an allelic replacement event at the *aroQ* locus.

LD_{50} experiments and in vivo growth. BALB/c mice were pedigree bred and maintained in the Department of Microbiology, University of Melbourne, Melbourne, Parkville, Australia. For 50% lethal dose (LD_{50}) experiments, groups of five sex- and age-matched mice were infected intraperitoneally with serial 10-fold dilutions of *C. pseudotuberculosis* in saline. The infecting dose was calculated retrospectively by viable count on BHI agar. Mice were killed by cervical dislocation when moribund. The LD_{50} value was calculated by the method of Reed and Muench (35) at the end of 8 weeks. The kinetics of bacterial growth in vivo was evaluated by sacrificing groups of four mice at regular time intervals postinfection. Spleens and livers were homogenized in a blender (Stomacher 80) Seward Medical, London, England), and the bacterial load was enumerated by viable count on BHI agar.

129/Sv/Ev mice of either sex homozygous for a disrupted gamma interferon receptor (IFN-γR) gene (IFN-$\gamma R^{-/-}$) and for null mutation (IFN-$\gamma R^{+/+}$) were produced as described previously (19) and bred at the John Curtin School of Medical Research, Australian National University, Canberra, Australia.

Immunization and wild-type challenge. Groups of 12 BALB/c mice were immunized intraperitoneally with 10-fold serial doses of CS100, TB521, or heat-killed bacteria. The viable count of the immunizing inoculum was determined retrospectively. Bacteria were heat killed by incubation at 60°C for 30 min. Verification of killing was determined by viable count. All mice, including naive controls, were challenged intraperitoneally 21 days postvaccination with an infectious dose of the wild-type strain. Groups of challenged mice were sacrificed on days 7 and 14 postchallenge, and organs were removed for bacterial culture.

Cytokine induction and IFN-γ ELISA. Spleens from immunized mice were removed aseptically, and single-cell suspensions were prepared by passage through wire sieves. Erythrocytes were removed by treatment with 0.017 M Tris-ammonium chloride, washed twice, and suspended in RPMI (CSL Ltd., Melbourne, Victoria, Australia) containing 10% fetal bovine serum, 2 mM L-glutamine, 1 mM pyruvate, 5×10^{-5} M β-mercaptoethanol, penicillin (100 U/ml), and streptomycin (50 μg/ml). Bulk splenocytes were seeded at 5×10^6/ml in 0.5 ml in 48-well tissue culture plates (Costar, Cambridge, Mass.). Cells were stimulated with 5 μg of soluble *C. pseudotuberculosis* cell lysate and incubated for 48 h before supernatant fluids were harvested. Control wells were stimulated with concanavalin A (5 μg/ml; Sigma, St. Louis, Mo.) or medium alone. Supernatant fluids were used in a IFN-γ cytokine enzyme-linked immunosorbent assay (ELISA) (CSL Ltd.) which was performed according to the manufacturer's instructions. The limit of detection in the assay was 2 IU/ml.

Statistics. The mean number of challenge bacteria recovered from immunized mice was compared to the number recovered from unvaccinated mice by using ordinary one-way analysis of variance with Dunett's analysis. The unpaired Student *t* test was used for comparison of bacterial counts from IFN-$\gamma R^{-/-}$ mice with control mice.

Nucleotide sequence accession number. The complete nucleotide sequence of the *C. pseudotuberculosis* C231 *aroB* and *aroQ* genes has been lodged in GenBank under accession no. U88628.

RESULTS

Cloning of genes from the prechorismate aromatic amino acid biosynthetic pathway. Initial attempts to clone the *C. pseudotuberculosis aroA* gene by shotgun cloning 2- to 4-kb *Sau*3A-digested genomic DNA fragments and identification of recombinants which could complement the *aroA E. coli* mutant AB2829 proved unsuccessful. To increase the likelihood of obtaining a representative genomic library, a cosmid library was constructed in the vector pHC79. The library was amplified in the lambda lysogen BRD728 by incubation at 37°C, and the lysate containing a cosmid bearing defective phage particles was used to independently transduce each of the prechorismate *E. coli aro* mutants AB2829, AB2826, AB2830, and AB1360. Complementation of growth on minimal medium was achieved only for the *aroB* and *aroD E. coli* mutants, AB2826 and AB1360, respectively. Restriction enzyme digests of complementing cosmids isolated from both mutants suggested the presence of many shared restriction fragments. Indeed, all analyzed cosmids that complemented AB2826 could also complement AB1360.

One such cosmid (pCS1) was subcloned to a 3-kb *Bam*HI fragment in pBluescript KS^+. The resultant construct, pCS2, retained the ability to complement growth of both AB2826 and AB1360. Further subcloning and sequencing of pCS2 identified two open reading frames. BLAST analysis of the DNA and predicted amino acid sequences of the two open reading frames indicated most significant amino acid identity with the *aroB* and *aroQ* gene products from *M. tuberculosis*. These shikimate pathway genes encode the enzymes dehydroquinate synthase and a type II 3-dehydroquinase, respectively. On the basis of sequence identities, these genes from *C. pseudotuberculosis* were designated *aroB* and *aroQ*. The deduced amino acid sequences of the *aroB* and *aroQ* genes from *C. pseudotuberculosis* were aligned with the amino acid sequences of other *aroB* and *aroQ* genes by using the CLUSTAL multiple alignment program (Australian Genomic Information Service). The deduced amino acid sequence of the *aroB* gene displayed significant identity with *aroB*-encoded enzymes from other bacterial species (Fig. 1A). The deduced amino acid sequence of the *aroQ* gene, while displaying significant identity with the *M. tuberculosis aroQ* gene product, also displayed identity with catabolic enzymes involved in quinic acid catabolism in fungi (Fig. 1B).

Construction of *C. pseudotuberculosis aroQ::erm*. The observation that ColE1-based plasmids such as pBluescript KS^+ cannot replicate in *C. pseudotuberculosis* facilitated the construction of a suicide targeting vector designed to mutate the *aroQ* gene. The construction of pCS6 (targeting vector) and pCS7 (control plasmid) is outlined in the Materials and Methods. Importantly, pCS6 could not complement the growth of *aroD E. coli* mutant AB1360 on minimal media. This result suggested that the product of the cloned *aroQ* gene was now no longer functional. Electroporation of pCS6, but not the control plasmid pCS7, into *C. pseudotuberculosis* C231 and TB521 yielded erythromycin-resistant colonies of various sizes after 4 days of incubation at 37°C. These colonies were presumed to have resulted from a recombination event. Erythromycin-resistant colonies were then patched onto BHI agar containing kanamycin. Of the erythromycin-resistant colonies generated, approximately 5% were kanamycin sensitive.

Unlike the situation with many gram-negative pathogens, the lack of a defined minimal medium for culture of *C. pseudotuberculosis* prevented screening putative *aroQ* mutants for aromatic amino acid auxotrophy. As a means of verifying allelic exchange, chromosomal DNAs from putative mutants and the wild-type strain were analyzed by Southern hybridization with probes specific for *aroB* and *-Q* and the erythromycin resistance gene. The strategy used to construct and analyze these mutants is represented schematically in Fig. 2A.

As predicted, a 3.8-kb *Bgl*II fragment in representative *aroQ* mutants of C231 and TB521 hybridized to the 2-kb *Bgl*II *aroB,Q*-specific probe (Fig. 2B). The gel shift of 1.8 kb in the mutants relative to the band in the wild-type parental strain corresponds to the size of the erythromycin resistance cassette inserted into the *aroQ* gene. After stripping of the *aroB,Q*-specific probe, an identically sized 3.8-kb *Bgl*II fragment in DNA extracted from the putative mutants hybridized to an erythromycin resistance gene-specific probe (Fig. 2C). With this probe, there was no hybridization to DNA extracted from the wild-type strain. Labeled pBluescript DNA did not hybridize to DNA from either the wild-type strain or the representative mutants (data not shown). The results indicate legitimate allelic exchange at the *aroB* and *-Q* loci, resulting in the construction of an *aroQ* mutant of C231, designated CS100, and also of TB521, designated CS200.

In vitro growth characteristics. Initial observations on the growth characteristics of CS100 and CS200 suggested that they grew more slowly in vitro than their parental counterparts. Importantly, however, the growth rate could be restored by providing the cloned *aroQ* gene on a plasmid in *trans* (Fig. 3). These results suggested that the reduced growth rate displayed by the *aroQ* mutants was not a pleiotropic effect caused by introduction of the *aroQ* mutation but rather more likely re-

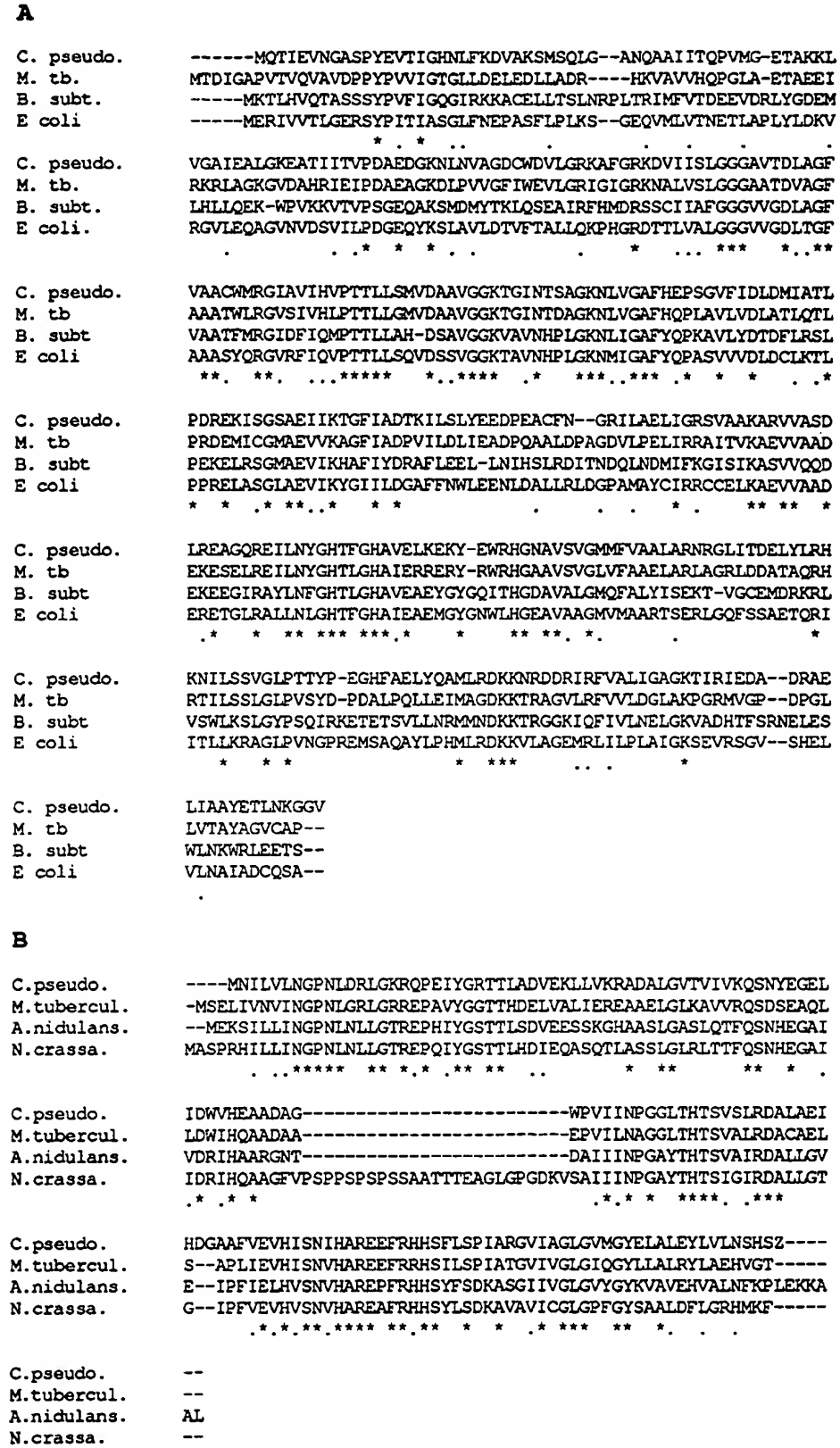

FIG. 1. Amino acid comparisons of *aroB* and *aroQ* gene products. (A) Comparison of deduced amino acid sequences of *aroB*-encoded dehydroquinate synthase enzymes from *C. pseudotuberculosis* (*C. pseudo.*), *M. tuberculosis* (*M. tb.*), *E. coli*, and *B. subtilis* (B. subt.). (B) Comparison of deduced amino acid sequences of *aroQ*-encoded 3-dehydroquinase enzymes from *C. pseudotuberculosis* and *M. tuberculosis* (M. tubercul.) in optimal alignment with the catabolic 3-dehydroquinases from *Aspergillus nidulans* and *Neurospora crassa*. The alignment was made by using the CLUSTAL DNA alignment program made available through the Australian Genomic Information Service. In each alignment, residues that are identical between species are indicated by asterisks. Conservative substitutions are indicated by dots.

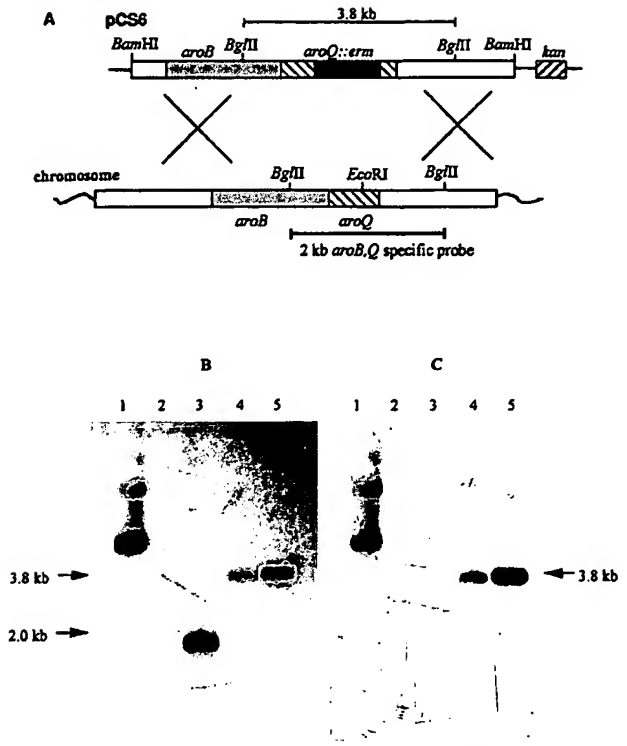

FIG. 2. The construction of *aroQ* mutants of *C. pseudotuberculosis*. (A) Schematic diagram illustrating the strategy used to generate *aroQ* mutants and derive probes for screening recombinants. (B and C) Results of Southern hybridization of chromosomal DNA extracted from C231, CS100, and CS200, digested with *Bgl*II, and probed with (B) an α-^{32}P-labeled 2-kb *BglII aroB,Q*-specific gene probe (B) and an α-^{32}P-labeled erythromycin resistance gene. Lanes: 1, uncut pCS6; 2, empty lane; 3, C231; 4, CS100; 5, CS200.

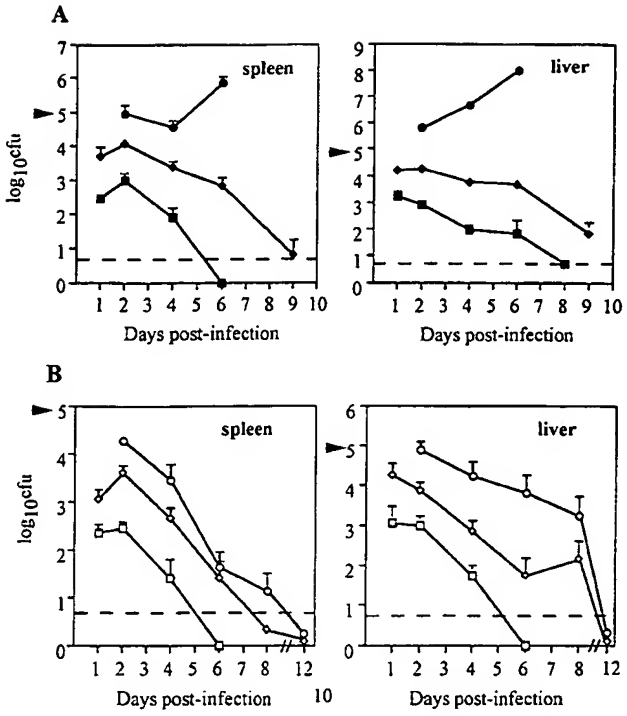

FIG. 4. *aroQ* mutants fail to persist in vivo. (A) In vivo growth of C231 (●), CS100 (■), and CS100(pCS8) (◆) in spleens and livers of BALB/c mice after intraperitoneal injection of 10^5 bacteria of each strain. (B) In vivo growth of TB521 (○), CS200 (□), and CS200(pCS8) (◇) in spleens and livers of BALB/c mice after intraperitoneal injection of 10^5 bacteria of each strain. In all cases, each point represents the mean of four mice plus the standard deviation. The detection limit (dashed line) was 5 CFU/organ.

flects the inability of *C. pseudotuberculosis* to scavenge one or more key aromatic metabolites from the growth medium.

***aroQ* mutants of *C. pseudotuberculosis* are attenuated in a mouse model of infection.** Introduction of the *aroQ* mutation into *C. pseudotuberculosis* C231 increased the LD_{50} for BALB/c mice by ~3 logs (increase in the $\log_{10}$ LD_{50} value from 2.5 to 5.3). In contrast, introduction of the *aroQ* mutation into TB521 attenuated this strain by only a further log relative to the attenuation already caused by mutation of *pld* (increase in the $\log_{10}$ LD_{50} value from 5.3 to 6.3). Mice which succumbed to infection with high doses of either *aroQ* mutant died within 24 to 48 h. However, death appeared to be associated with direct toxicity and not bacteremia, since, as determined by bacterial culture of organs, there was no significant expansion of bacterial numbers in vivo. These experiments identified the maximum tolerated dose for BALB/c mice as ~10^6 CFU.

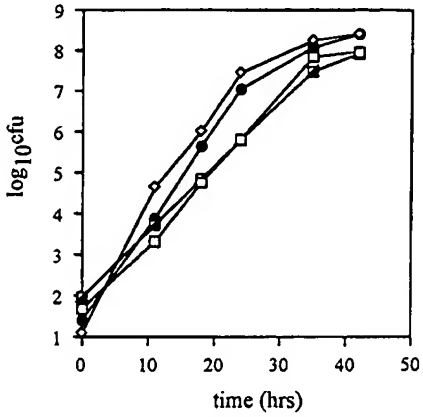

FIG. 3. In vitro growth rates of C231 (●), CS100 (□), CS100(pEP-2) (), and CS100(pCS8) (◇) in BHI broth. The results are representative of two separate experiments.

Persistence of *aro* mutants and parental counterparts in vivo. To better understand the basis for the observed attenuation, we examined the degree of in vivo persistence of CS100, CS200, and their parental strains in sublethally infected mice (Fig. 4A and B). The results demonstrated that introduction of an *aroQ* mutation severely restricted the in vivo growth of the mutants compared with their parental counterparts. Bacteria harboring an *aroQ* mutation, regardless of parental background, could not be cultured from spleens and livers of infected mice beyond 8 days postinfection. In contrast, mice infected with wild-type strain C231 harbored a significant bacterial burden in spleens and livers and were moribund by day 6 postinfection (Fig. 4A). Mice infected with TB521 harbored bacteria in spleens and livers, albeit in reduced numbers, until at least day 8 postinfection (Fig. 4B). These mice did not display any clinical symptoms. When the *aroQ* mutants were complemented in *trans* with an intact *aroQ* gene, the in vivo persistence of both CS100 and CS200 was increased but remained less than that of either parental strain.

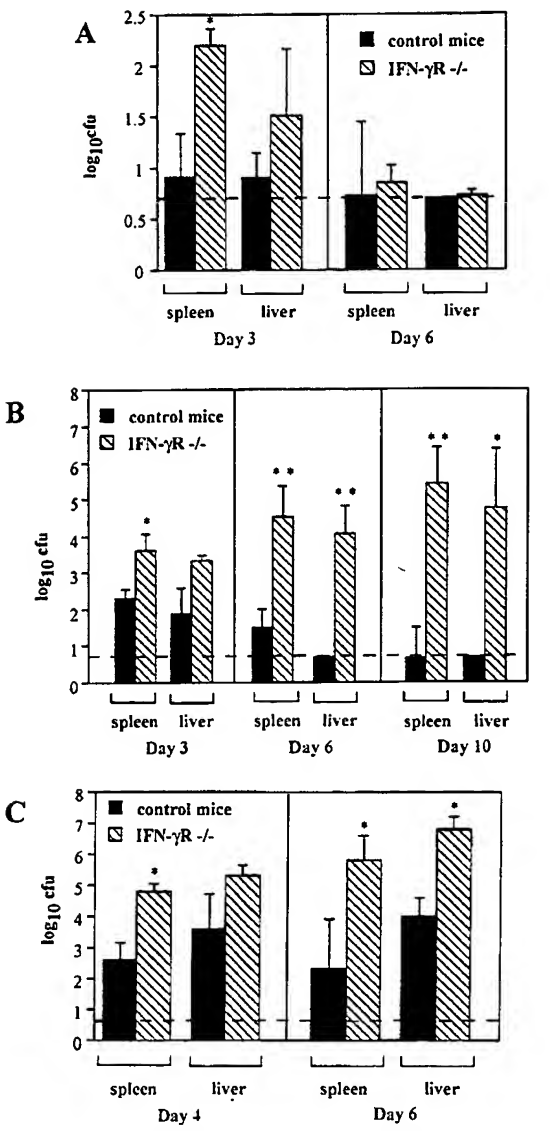

FIG. 5. In vivo growth of CS100 (A), TB521 (B), and C231 (C) in IFN-γR$^{-/-}$ and homozygous control mice. Mice were intraperitoneally infected with 5×10^4 CFU of CS100, 10^4 CFU of TB521, and 10^4 CFU of C231. Each bar represents the mean organ count from four mice plus the standard deviation. The detection limit (dashed line) was 5 CFU/organ. * denotes $P < 0.01$, and ** denotes $P < 0.001$, (Student's t test) compared to homozygous control mice.

***aroQ* mutants are attenuated in IFN-γR$^{-/-}$ mice.** A requirement of live attenuated vaccines is that they should be safe even when used in immunocompromised hosts. In relation to this, the importance of the cytokine IFN-γ in controlling growth of both intracellular bacteria and viruses is well documented. We have used gene knockout mice which lack the receptor for IFN-γ to determine whether this cytokine has a role in mediating clearance of *C. pseudotuberculosis* in vivo. At the infectious dose given, the overall kinetics of infection with CS100 in IFN-γR$^{-/-}$ mice was not greatly different from that in homozygous control mice (Fig. 5A). This result suggests that IFN-γ plays only a minor role in mediating clearance of an aromatic mutant of *C. pseudotuberculosis*.

In direct contrast, IFN-γR$^{-/-}$ mice infected with either the *pld* mutant TB521 (Fig. 5B) or the wild-type strain, C231 (Fig. 5C) were highly susceptible to infection compared to homozygous control mice. As a control for the phenotype of these mice, animals were also infected with an *S. typhimurium* Δ*aroA* Δ*aroD* mutant (BRD509); in accordance with published results (12), IFN-γR$^{-/-}$ mice were more susceptible to infection than control mice (data not shown).

Protection against *C. pseudotuberculosis* wild-type challenge. Groups of six BALB/c mice were immunized intraperitoneally with graded sublethal doses of either CS100, TB521, or heat-killed CS100. All mice, including naive controls, were subsequently challenged 21 days postvaccination with an infectious dose (5×10^3 CFU) of the wild-type strain. Groups of challenged mice were sacrificed on days 7 and 14 postchallenge, and organs were removed for bacterial culture of the wild-type strain (Fig. 6). Protection was assessed by comparison of the bacterial load in naive mice to that in immunized mice. At 14 days postchallenge, the absence of challenge bacteria in the organs of mice immunized with 5×10^5 or 5×10^4 CFU of TB521 indicated the development of protective immunity in all of these animals. Similarly, mice immunized with the maximum tolerated dose of CS100 (8×10^5 CFU) harbored significantly fewer challenge bacteria at both days 7 and 14 postchallenge compared to naive animals. In contrast, mice which received 8×10^4 CFU of CS100, 1×10^6 CFU of heat-killed bacteria, or no vaccine were not protected from challenge and harbored significant bacterial burdens in both the spleen and liver at days 7 and 14 postinfection.

Induction of IFN-γ by vaccine strains. At day 7 postvaccination, IFN-γ could be detected in supernatant fluids of stimulated splenocytes derived from mice vaccinated with 8×10^5 CFU of CS100 or either dose of TB521. Thus, there was a qualitative correlation between detectable IFN-γ production

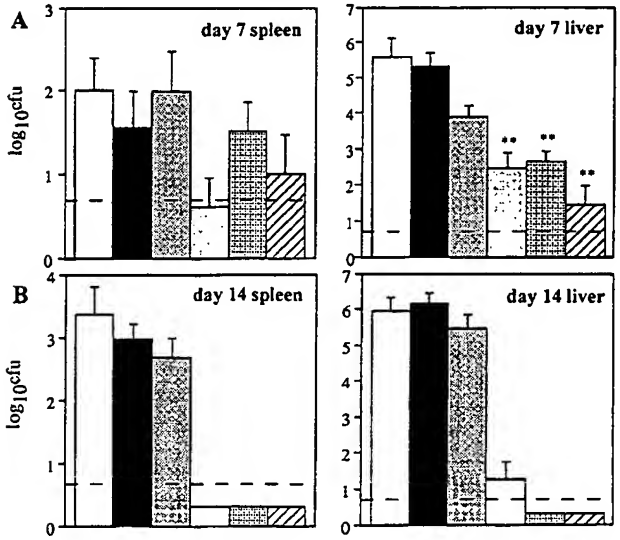

FIG. 6. Capacity of attenuated mutants of *C. pseudotuberculosis* to protect mice from a lethal homologous challenge. Groups of six mice were not vaccinated (□), or vaccinated intraperitoneally at day 0 with 10^6 heat-killed CS100 (■), 8×10^4 CFU of CS100 (■), 8×10^5 CFU of CS100 (□), 5×10^4 CFU of TB521 (▦), or 5×10^5 CFU of TB521 (▨). by the same route at day 21 with 5×10^3 CFU of the wild-type strain. Groups of mice were sacrificed, and organs were removed for bacterial culture of the challenge strain on day 7 (A) and day 14 (B) postchallenge. Each bar represents the mean organ count from spleens and livers of six mice plus the standard deviation. The detection limit (dashed line) was 5 CFU/organ. ** denotes $P < 0.01$ (analysis of variance) compared to unvaccinated mice.

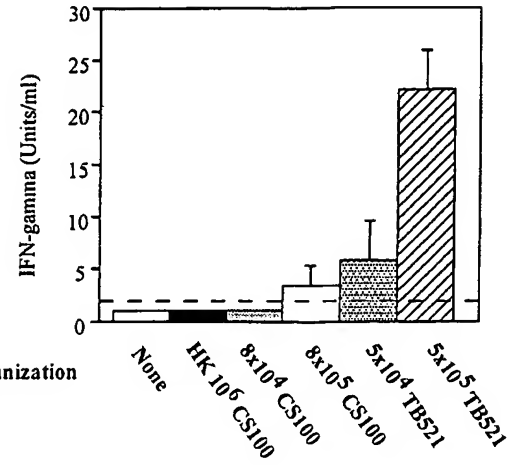

FIG. 7. IFN-γ detection in supernatant fluids of cultured mouse splenocytes isolated and pooled from vaccinated mice. Splenocytes were prepared 7 days postvaccination and stimulated with 5 μg of *C. pseudotuberculosis* soluble antigen. The detection limit of the cytokine ELISA was 2 IU/ml (dashed line).

by antigen-stimulated splenocytes derived from vaccinated mice and subsequent protection from challenge (Fig. 7). IFN-γ was not detected in supernatant fluids from stimulated splenocytes at day 14 postvaccination.

DISCUSSION

In this report, we describe the construction of *C. pseudotuberculosis* strains having insertion mutations in the *aroQ* gene and subsequent preliminary in vivo characterization. While *aro* mutants of other gram-positive bacteria have been constructed by transposon mutagenesis (1, 20), this is, to our knowledge, the first report of a rationally attenuated *aro* mutant of a gram-positive bacterial pathogen generated through allelic exchange.

Historically, *aro* genes from pathogenic bacteria have been cloned by complementation of growth of defined *E. coli aro* mutants on minimal media. This complementation approach has been highly successful in obtaining genes encoding aromatic biosynthetic enzymes from gram-negative bacterial pathogens. Using this same approach, we were able to identify cosmids of *C. pseudotuberculosis* DNA which complemented *aroB* and *aroD E. coli* mutants but not cosmids which complemented *aroA* or *aroC* mutants. This may reflect the potentially nonrepresentative nature of the cosmid library. Alternatively, it may reflect a significant degree of divergence between the *E. coli aroA*- and *aroC*-encoded enzymes and the aromatic pathway enzymes from *C. pseudotuberculosis.*

The sequential genetic arrangement of *aroB* and *aroQ* in *C. pseudotuberculosis*, as established in this study, corresponds to the order in which homologous genes are found in the taxonomically related *M. tuberculosis* (7). Moreover, the significant identity at the deduced amino acid level between the products of the *C. pseudotuberculosis* housekeeping genes *aroB*, *aroQ*, and *recA* (33), the *groEL* and *dnaK* gene products (unpublished data), and the corresponding gene products from *M. tuberculosis* confirms their relatedness at the molecular level.

Despite the amino acid sequence relatedness of the *C. pseudotuberculosis aroQ*-encoded 3-dehydroquinase enzyme to fungal catabolic enzymes from *Neurospora crassa* (8) and *Aspergillus nidulans* (11), there are several lines of reasoning in support of its involvement in the *C. pseudotuberculosis* aromatic amino acid biosynthetic pathway. First, of the analyzed recombinant cosmids and subclones capable of complementing growth of the *aroD E. coli* mutant on minimal media, virtually all displayed identical restriction enzyme digest patterns, suggesting that there is only one gene encoding a 3-dehydroquinase enzyme in *C. pseudotuberculosis*. Second, the close genetic linkage of the *aroQ* gene to the *aroB* gene, which, in *E. coli*, encodes an enzyme catalyzing the preceding step of the aromatic biosynthetic pathway, similarly suggests a biosynthetic function for the *aroQ* gene product. Third, there is evidence that *aroQ* homologs from other procaryotes, including the closely related *M. tuberculosis*, do not encode enzymes with catabolic activity (7, 25).

Random recombination of transformed DNA, as occurs in slow-growing mycobacteria, does not appear to be an impediment to the construction of mutants of *C. pseudotuberculosis* by allelic exchange. The construction of *aroQ* mutants of *C. pseudotuberculosis* was, however, problematic, since such mutants unexpectedly displayed a reduced growth rate in vitro on complex media. Importantly, however, the in vitro growth rate could be restored to wild-type levels by introduction of the intact *aroQ* gene in trans. The *aroQ* mutants generated in this study were significantly attenuated in a murine model of infection. *C. pseudotuberculosis aroQ* mutants CS100 and CS200 were cleared from livers and spleens of intraperitoneally infected mice by day 8 postinfection. While complementation in *trans* with an intact *aroQ* gene increased their in vivo persistence, it did not fully restore virulence to wild-type levels. This may, in part, have been due to plasmid segregation in vivo. Mice infected with a lethal dose of either *aroQ* mutant died within 48 h; there was not a significant expansion of bacterial numbers in either the spleen or liver, which suggested that toxicity was the cause of death.

The in vivo growth kinetics of *C. pseudotuberculosis aroQ* mutants in BALB/C mice contrasts with the behavior of *S. typhimurium aroA* mutants, which can persist in vivo for several weeks after intravenous infection (28). However, it would appear that *S. typhimurium aroA* mutants are exceptional with respect to their in vivo persistence, since *aroA* mutants of *B. pertussis* (36), *A. salmonicida* (42), *P. multocida* (18), and *Y. enterocolitica* (4) are also rapidly cleared from major organs of experimentally infected animals. Indeed, the capacity of aromatic mutants of *S. typhimurium* to kill IFN-γR$^{-/-}$ mice suggests that aromatic metabolites are not limiting in vivo and that bacterial clearance is at least partially dependent on the host's immune response. That *aroQ* mutants of *C. pseudotuberculosis* were attenuated in IFN-γR$^{-/-}$ mice suggests, not surprisingly, fundamental differences in bacterial physiology and pathogenesis between *S. typhimurium* and *C. pseudotuberculosis.*

Given the relatedness of *C. pseudotuberculosis* to *M. tuberculosis*, it is interesting to speculate on the likely phenotype of *aro* mutants of *M. tuberculosis*. The prechorismate genes *aroA*, *aroB*, and *aroQ* have been cloned from *M. tuberculosis* and represent suitable targets for mutagenesis. In light of our findings, we predict that should rational *aro* mutants of *M. tuberculosis* be constructed, they will be highly attenuated. By using cosmid DNA to mediate site-specific allelic exchange, the construction of defined mutants of *M. tuberculosis* strains now appears possible (2).

The immune mechanisms mediating clearance of CS100 in vivo remain undefined. While IFN-γR$^{-/-}$ mice have the capacity to mount a Th1-type T-cell response, macrophage activation is largely abrogated (19, 38, 39). Since IFN-γR$^{-/-}$ mice adequately controlled infections with CS100, bacterial clearance of CS100 may not be critically dependent on IFN-γ-activated macrophages. The absence of activated macrophages

does not, however, preclude the induction of acquired immunity via the two primary sources of IFN-γ, T lymphocytes and NK cells. Importantly, from a vaccine vector point of view, the results suggest that infection with CS100 is largely self-limiting, even in immunocompromised animals. On the other hand, IFN-γR$^{-/-}$ mice were highly susceptible to primary infections with either *C. pseudotuberculosis* C231 or the *pld* mutant TB521 compared to control mice. Thus, control of these primary *C. pseudotuberculosis* infections, as with infections caused by other intracellular bacterial pathogens, is significantly dependent on the bacteriocidal capacity of activated macrophages to contain and destroy the pathogen. Concordantly, Hard (9) has shown a role for T lymphocytes and activated macrophages in suppression of *C. pseudotuberculosis* growth and lesion development in a murine model.

Despite the lower level of in vivo persistence of CS100 than of TB521, this strain retained the capacity to elicit a protective immune response in BALB/c mice. The induction of protective immunity was dose dependent, however, since mice immunized with 8×10^4 CFU of CS100 were not protected from challenge. In these experiments, the induction of protective immunity by TB521 was not dose dependent, since mice immunized with either 5×10^5 or 5×10^4 CFU of TB521 were completely protected. Thus, we hypothesize that the enhanced capacity of TB521 to elicit a protective immune response can be attributed to its greater in vivo persistence. As a consequence, this strain may have an increased capacity to stimulate IFN-γ-producing T cells and/or NK cells. Indeed, appropriate T-cell stimulation is considered an essential requirement for acquired resistance against most intracellular pathogens (24). The observation that mice immunized with 10^6 heat-killed *C. pseudotuberculosis* were not protected from challenge supports the contention that a live vaccine is better able to stimulate the appropriate protective immune response. Indeed, with one exception (40), the use of killed bacterial preparations as vaccines against intracellular bacterial pathogens has historically been relatively unsuccessful (5, 6).

The role of the humoral immune response in mediating acquired resistance to *C. pseudotuberculosis* challenge remains incompletely defined. At the time of challenge, antibodies to *C. pseudotuberculosis* whole-cell lysate could not be detected in any CS100-vaccinated mice (data not shown), despite the immune status of these mice. This finding suggests that the presence of pathogen-specific circulating antibodies is not essential for protection against *C. pseudotuberculosis* challenge in a mouse model.

The use of live, rationally attenuated bacterial pathogens as vaccines, and as vaccine vectors, represents an attractive means of relatively safe, cheap, long-lasting, and efficacious vaccination, particularly within a veterinary context. We envisage that an *aroQ* mutant of *C. pseudotuberculosis*, as described here, represents a potential live vaccine and/or vaccine vector in sheep, since significantly more bacteria (up to 10^{10}) can be administered than in mice. Studies addressing these issues are currently in progress.

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Effect of Attenuated *Erysipelothrix rhusiopathiae* Vaccine in Pigs Infected with Porcine Reproductive Respiratory Syndrome Virus

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ABSTRACT. Twenty 2nd specific pathogen-free pigs were divided into 4 groups: Group A were infected with porcine reproductive and respiratory syndrome (PRRS) virus at 6 weeks of age and treated with available swine erysipelas and swine fever combined vaccine (vaccinated) at 7 weeks of age; Group B were vaccinated at 7 weeks of age and infected with PRRS virus at 8 weeks of age; Group C were vaccinated at 7 weeks of age: Group D were neither vaccinated nor infected with PRRS virus. All pigs were challenged to *Erysipelothrix rhusiopathiae* C42 strain at 10 weeks of age. No clinical signs appeared after vaccination of group A and B pigs, thus confirming that the safety of the vaccine was not influenced by infection with PRRS virus. None of the pigs in Groups A and C developed erysipelas after challenge exposure to *E. rhusiopathiae*. In contrast, fever and/or urticaria appeared transiently in all pigs of Group B after challenge exposure. At the time of challenge exposure to *E. rhusiopathiae*, the PRRS virus titer was high in sera of Group B, but was low in those from Group A. However, vaccination of pigs with attenuated *E. rhusiopathiae* was effective in dual infection with PRRS virus and *E. rhusiopathiae*, because the clinical signs were milder and the *E. rhusiopathiae* strain was less recovered from these pigs compared to pigs of group D. — KEY WORDS: *Erysipelothrix rhusiopathiae*, experimental infection, PRRS, swine, vaccine.

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Erysipelothrix rhusiopathiae is a causative pathogen for swine erysipelas, which causes enormous economic losses in pig production. The clinical signs of swine erysipelas can be divided into three types: acute (septicemia), subacute (urticaria), and chronic (arthritis and endocarditis). Lyophilized live vaccine prepared from an acriflavine-fast attenuated *E. rhusiopathiae* [15] has been widely used for prevention of swine erysipelas in Japan.

Reproductive disturbance in sows and respiratory distress in growing pigs were encountered around 1987 due to the outbreaks of porcine reproductive and respiratory syndrome (PRRS), and PRRS virus was isolated in 1993 [6, 12]. It has been believed that PRRS was associated with other microbial infections in growing pigs [10, 12]. Since 1988, in several large-scale pig farms infiltrated with PRRS virus, swine erysipelas broke out frequently even in 2–6-month-old pigs that had been vaccinated with commercially available attenuated *E. rhusiopathiae* vaccine (Hara, 1996, unpublished data). It was, therefore, suspected that the efficacy of the attenuated *E. rhusiopathiae* vaccine was diminished by PRRS virus infection in pig. The object of this study was to determine whether PRRS virus infection in pigs inhibited the effect of the attenuated *E. rhusiopathiae* vaccine.

MATERIALS AND METHODS

Pigs: Twenty 6-week-old 2nd specific-pathogen-free (SPF) pigs (produced at the Zen-Noh Central Institute for Feed and Livestock Research) were used. The pigs were separated into groups which were housed separately in clean pig rooms.

Vaccine: A commercially available swine erysipelas and swine fever combined live vaccine (Scientific Feed Laboratory Co., Ltd., Tokyo, Japan) was used. The reconstituted vaccine contained acriflavine-fast attenuated strain Koganei 65–0.15 of *E. rhusiopathiae* ($\geqq 1 \times 10^8$ viable bacteria/m*l*) [15] and strain GPE of hog cholera virus ($\geqq 10^3$ median tissue culture infective dose ($TCID_{50}$)/m*l*) [7].

Cell cultures: Swine alveolar macrophage (SAM) [3, 16] cells obtained from 4- to 6-week-old SPF pigs, were used throughout this experiment. A cell line derived from rhesus monkey kidney, MARC-145 cells [4] which was kindly provided by Murakami (National Institute of Animal Health, Japan), was also used for serological examination.

Virus and bacterial strains: PRRS virus strain E4 and *E. rhusiopathiae* strain C42 were used for challenge exposure of swine. PRRS virus strain E4 was isolated from a severely affected pig in 1993 [9]. Strain E4 was grown in fresh cultures of SAM cells at 37°C in 5% CO_2 incubator, and was diluted to a $10^{5.8}$ $TCID_{50}$/m*l*.

E. rhusiopathiae strain C42 of serotype 1a was isolated from a growing pig which was dying due to dual infection with PRRS virus and *E. rhusiopathiae* in 1995. Strain C42 was grown on nutrient broth (Eiken Chemical Co., Ltd., Tokyo, Japan), and adjusted to $10^{6.5}$ colony-forming-units (CFU)/m*l*.

Experimental design: The pigs were divided into 4 groups (Table 1). Pigs of Groups A–C were injected subcutaneously with a commercially available swine erysipelas and swine fever combined vaccine at 7 weeks of age. All pigs were challenge-exposed to 0.1 m*l* of bacterial suspension of *E. rhusiopathiae* C42 strain administered intracutaneously in the side at 10 weeks of age (post-vaccination week (PVW) 3). A 1 m*l* suspension of PRRS

Table 1. Experimental design

Group	Pig No.	Passage of week 0[a]	1	2	3	4	5	6
A	51–55	PRRS[b]	Vac[c]			*E. r*[d]		Necropsy
B	56–60		Vac	PRRS		*E. r*		Necropsy
C	61-65		Vac			*E. r*		Necropsy
D	66–70					*E. r*		Necropsy

a) Six weeks of age. b) Inoculated with PRRS virus. c) Treated with swine erysipelas and swine fever combined vaccine. d) Challenged with *E. rhusiopathiae*.

virus was introduced intranasally at 6 weeks of age (pre-vaccination week 1) to pigs of group A, and at 8 weeks of age (PVW 1) to pigs of group B.

Clinical observation: The pigs were observed daily for body temperature, clinical signs of PRRS, erysipelas and other abnormalities.

Serological tests: Blood samples were collected from all pigs at intervals of one week to determine the antibody titers of serum. The indirect immunofluorescence assay (IIFA) test of PRRS virus [6] and growth agglutination (GA) test of *E. rhusiopathiae* [8] were carried out by methods described previously.

Recovery of PRRS virus: The SAM cells suspended in Eagle's minimum essential medium supplemented with 10% of fetal calf serum (FBS-MEM) at $10^{6.2}$ cells/m*l* were seeded in 96-well plates in aliquots of 0.1 m*l* per well. Two hr after seeding, the cell cultures were inoculated with 10% tissue homogenates or 0.05 m*l* undiluted serum. After adsorption at 37°C for 1 hr, each well was washed twice with FBS-MEM and then fed with 0. 1 m*l* of fresh FBS-MEM. The plates were incubated at 37°C in a 5% CO_2-incubator for 6 days. The supernatant was inoculated onto fresh cultures of SAM cells if a cytopathic effect (CPE) was not observed during the first passage. Virus titration was performed by the microtiter method using the SAM cell cultures prepared as described above.

Recovery of E. rhusiopathiae: Specimens of the heart, lung, liver, spleen, kidney, lymphocentrun subiliacum and cutis (segment of challenged to *E. rhusiopathiae*) were plated on Tryptic Soy (TS) agar (Difco Lab., Detroit, MI., U.S.A.) containing 10% sheep blood and TS agar containing 5% horse serum, 50 μg/m*l* of gentamicin (GM) and 500 μg/m*l* of kanamycin (KM) (selective agar of *E. rhusiopathiae*), and were incubated for 2 days at 37°C. Swabs of the tonsil, hip joint and genu joint were cultured using TS broth (Difco Lab.,) containing 0.1% Tween 80, 50 μg/m*l* of GM and 500 μg/m*l* of KM and were incubated for 2 days at 37°C, and the culture medium was transferred to selective agar of *E. rhusiopathiae*, and was incubated for 2 days at 37°C. Suspected colonies of *E. rhusiopathiae* were identified using conventional biochemical tests [17]. The isolates were tested for resistance to acriflavine and pathogenicity in mice [15].

Necropsy findings: The pigs were sacrificed by intravenous overdose of thiopental sodium (Tanabe Pharmaceutical Co., Ltd., Osaka, Japan) at 12 weeks of age.

RESULTS

Clinical signs: Two or 3 pigs showed a local skin reaction at the site of vaccination, but no clinical signs or fever resulted from vaccination in all pigs of Groups A, B and C. In all pigs of Groups A and B, transient fever above 40°C was observed after inoculation with the PRRS virus.

After-challenge exposure to *E. rhusiopathiae*, fever occurred in all pigs with the exception of pigs in Groups A and C. In Groups B and D, urticaria was noted in 2 of the 5 pigs, respectively. On the other hand, no clinical signs were recognized in any pigs of Groups A and C (Table 2).

Serological tests: IIFA antibody to the PRRS virus was not detected in all pig sera collected before inoculation with PRRS virus, but was observed after inoculation in all pigs of Groups A and B, and the titers reached 1:40 to 1:320.

GA antibody to *E. rhusiopathiae* was not detected in all pigs at vaccination, but increased after vaccination in Groups A, B and C, up to geometric mean (GM) titers of 12.1, 9.2 and 10.6, respectively at PVW 3. The titers in these groups increased up to levels ranging from 24.3 to 32.0 at necropsy.

Recovery of PRRS virus: In Groups A and B, a large number of PRRS virus were recovered from sera of almost all pigs during post-inoculation weeks (PIW) 1 to 3, and a small amount of virus was recovered after PIW 4. At necropsy, the PRRS virus was recovered from the tonsil and/or lung of 2 pigs in Group A and all pigs of Group B. On the other hand, in Groups C and D, the PRRS virus was not recovered at any time from the serum, tonsil and lung of any pigs (Table 3).

Recovery of E. rhusiopathiae: At necropsy, *E. rhusiopathiae* was not recovered from any pigs of Groups A and C, but was recovered from 2 and 4 pigs in Groups B and D, respectively (Table 4). All of the recovered organisms were sensitive to acriflavine (minimal inhibitory concentration: 0.001–0.002% for all the isolates) and fatal to mice (50% lethal dose (LD_{50}): $10^{3.2}$–$10^{2.5}$ CFU), which were used in challenge exposure.

Necropsy findings: The necropsy findings in all pigs of Groups A, B and C were nearly normal. Two of the 5 pigs in Group D had muddy hip joint liquid.

DISCUSSION

Field evidence, strongly suggests a role of PRRS virus in predisposing animals to secondary infection [5, 10, 12]. In some cases, the pigs, which had already been vaccinated with attenuated *E. rhusiopathiae*, were found moribund due to acute swine erysipelas and dual infection with PRRS virus. The strain C42 of *E. rhusiopathiae* was isolated in these cases, and this strain was used for challenge in order to confirm the field evidence in this study. The field evidence aroused a doubt about the safety of attenuated *E. rhusiopathiae* vaccines in PRRS virus-infected pigs. The

Table 2. Body temperature and clinical signs in pigs after challenge with *E. rhusiopathiae*

Group[a]	Pig No.	Body temperature[b]	Urticaria on the skin[c]	Depression and loss of appetite[d]
A	51	–	–	–
	52	–	–	–
	53	–	–	–
	54	–	–	–
	55	–	–	–
B	56	40.6 (4)	–	–
	57	41.9 (5)	++ (4)	–
	58	41.9 (6)	+ (2)	+ (1)
	59	40.6 (4)	–	–
	60	40.3 (4)	–	–
C	61	–	–	–
	62	–	–	–
	63	–	–	–
	64	–	–	–
	65	–	–	–
D	66	40.1 (1)	–	–
	67	> 42.0 (6)	++ (6)	++ (8)
	68	40.6 (6)	–	+ (1)
	69	41.0 (3)	–	–
	70	> 42.0 (6)	++ (9)	++ (10)

a) See Table 1. b) Maximum temperature, –: < 40°C. ( ): Number of days with fever ≧40°C. c) –: No lesion, +: Lesion partially on body, ++: Lesion on whole body. ( ): Number of days with symptom. d) –: No clinical signs, +: Slight depression, ++: Severe depression and loss of appetite. ( ): Number of days with symptom.

Table 3. Recovery of PRRS virus from serum during six weeks and from tissues at necropsy

		Recovery of PRRS virus from serum							Recovery of PRRS virus at necropsy	
	Pig	passage of week								
Group[a]	No.	0	1	2	3	4	5	6	tonsil	lung
		↓				▼				
A	51	–[b]	4.30[c]	2.05	>4.80	2.05	–	–	–	–
	52	–	3.30	1.80	2.30	+[d]	+	+	3.05	+
	53	–	3.80	2.30	+	+	–	–	–	–
	54	–	4.30	+	2.80	+	–	–	–	–
	55	–	4.55	2.05	2.55	2.80	1.80	–	3.80	–
				↓		▼				
B	56	–	–	–	2.05	+	3.05	–	2.80	–
	57	–	–	–	3.80	3.30	2.55	–	3.30	2.80
	58	–	–	–	4.05	4.05	3.05	+	3.30	–
	59	–	–	–	4.30	2.55	3.05	+	4.05	4.30
	60	–	–	–	4.30	3.80	3.80	3.05	2.55	2.30

a) See Table 1. b) Negative. c) Log $TCID_{50}$/m*l*. d) Positive (< log 1.8 $TCID_{50}$/m*l*). ↓ Inoculated with PRRS virus. ▼ challenged with *E. rhusiopathiae* C42.

PRRS virus was also suspected of interfering with the effect of this attenuated vaccine. In this study, no clinical evidence of swine erysiplelas appeared after vaccination of pigs in Groups A and B, thereby confirming that a commercially available swine erysipelas and swine fever combined vaccine was safe and that its efficacy was not influenced by the inoculated PRRS virus. No pigs in Group A developed swine erysipelas after challenge exposure to the *E.*

Table 4. Recovery of *E. rhusiopathiae* from several tissues at necropsy

Group[a)]	Pig No.	Tonsil	Heart	Hip-joint	Others
A	51	–	–	–	–
	52	–	–	–	–
	53	–	–	–	–
	54	–	–	–	–
	55	–	–	–	–
B	56	–	–	–	–
	57	–	–	–	–
	58	–	+	–	–
	59	–	–	+	–
	60	–	–	–	–
C	61	–	–	–	–
	62	–	–	–	–
	63	–	–	–	–
	64	–	–	–	–
	65	–	–	–	–
D	66	–	–	–	–
	67	+	–	+	–
	68	+	–	–	–
	69	+	–	–	–
	70	–	+	+	+ (spleen, kidney, lymphocentrum,genu-joint)

a) See Table 1. –: Negative, +: Positive.

rhusiopathiae C42 strain. In contrast, transient fever and/or urticaria appeared in all pigs of Group B. At the time of challenge exposure to *E. rhusiopathiae* strain C42, PRRS virus recovered from sera of pigs in Group B had a high titer, but that in Group A had low titer. These results indicate that the outbreak of swine erysipelas is due to infection of virulent *E. rhusiopathiae* and severe PRRS viremia in pigs vaccinated with attenuated *E. rhusiopathiae*. However, vaccination of pigs with attenuated *E. rhusiopathiae* was effected by dual infection with PRRS virus and *E. rhusiopathiae*, because the clinical signs were milder and the *E. rhusiopathiae* strain, used in challenge exposure, was recovered in less amounts from these pigs, compared to pigs that were unvaccinated and infected with *E. rhusiopathiae* alone. It was suggested that immuno compromising occurred by infection with PRRS virus. However, little is known about the propensity of PRRS virus for alveolar macrophages [5, 11], and the suppression for systemic immunopathy is unknown.

All pigs of Group D (neither vaccinated nor treated with the PRRS virus) developed fever after challenge exposure to the *E. rhusiopathiae* C42 strain. However, clinical evidence of swine erysipelas was recognized in only 2 of 5 pigs. Takahashi *et al.* [13] reported that LD_{50} of a virulent strain of *E. rhusiopathiae* in inoculated mice was $<10^2$ CFU and that of avirulent strain $> 10^7$ CFU. LD_{50} of the C42 strain in inoculated mice was $10^{2.7}$ CFU (unpublished data). These results suggest that the C42 strain is a mesovirulent strain, and may indicate that even a mesovirulent strain of *E. rhusiopathiae* can cause acute swine erysipelas in pigs affected with PRRS in the field.

It has been reported that PRRS virus infection in pigs exacerbated bacterial diseases. For example, PRRS virus predisposes piglets to clinical disease caused by *Streptococcus suis* serotype 2 [2]. The interaction between PRRS virus and *Mycoplasma hyorhinis* in hysterectomy-produced and colostrum-deprived pigs has been recognized [12]. In other experimental studies, no differences have been found in clinical signs and lung lesions of pigs coinoculated with PRRS virus and *Mycoplasma hyopneumoniae* [14] or other bacteria [1]. Future studies on PRRS virus titers at secondary infection with microorganisms are awaited to define evidence contradictory to these reports.

It was considered that the susceptibility induced by PRRS virus to secondary diseases does not last for a long time and the risk period seems to be short, and limited to the time when the PRRS virus is remarkably proliferated in the affected pig. The results of this study support the safety and efficacy of the attenuated *E. rhusiopathiae* vaccine in pigs infected with PRRS virus.

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Cross protection of mice and swine inoculated with culture filtrate of attenuated *Erysipelothrix rhusiopathiae* and challenge exposed to strains of various serovars

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SUMMARY

Mice and swine inoculated subcutaneously with culture filtrate vaccine prepared from acriflavine-fast attenuated *Erysipelothrix rhusiopathiae* strain Koganei 65-0.15 (serovar 2), were challenge exposed to 20 pathogenic strains of *E rhusiopathiae* of 18 serovars and type N. Vaccinated mice survived after challenge exposure to serovars 1b, 2, 8 (strain Goda), and type N, but mortality occurred in vaccinated mice challenge exposed to other strains: 20% to 30% mortality in mice challenge exposed to serovars 1a, 11, 12, 15, 16, or 21; 40% to 50% mortality in mice challenge exposed to serovars 4, 5, 6, 7, or 8 (strain 911); and 60% to 80% mortality in mice challenge exposed to serovars 9, 10, 18, or 19. All vaccinated mice died after challenge exposure with strain 2553 (serovar 20). Nonvaccinated control mice died after challenge exposure to all strains.

Of 2 vaccinated swine challenge exposed to strain 2553, 1 developed a local urticarial lesion at the site of intradermal exposure. Vaccinated swine challenge exposed to serovars 1a, 1b, 2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 15, 16, 18, 19, 21, or type N did not have clinical signs of acute erysipelas. Nonvaccinated control swine developed acute generalized erysipelas or localized urticarial lesions at the site of intradermal exposure.

Strains of *Erysipelothrix rhusiopathiae* are classified into 22 serovars and type N. These serovars are based on antigenic characteristics of soluble peptidoglycans.[1,2] Most isolants of *E rhusiopathiae* from swine with clinical erysipelas are serovars 1a, 1b, and 2.[3-8] However, some isolants of relatively rare serovars 3, 4, 5, 6, 7, 8, 10, 11, 15, and 21 and type N have been obtained from swine with septicemia, urticaria, arthritis, lymphadenitis, and endocarditis.[5-8]

Some isolants of serovars 3, 4, 5, 6, 8, 9, 10, 11, 12, 13, 15, 17, 18, 19, and 20 and type N are pathogenic for swine,[9-13] indicating they could be a cause of clinical erysipelas.

Strains of *E rhusiopathiae* serovar 9 or 10 caused acute localized or generalized urticarial lesions in swine and fatal septicemia in mice vaccinated with standard erysipelas adsorbate bacterin prepared from strains of serovar 2.[14,15] Some swine vaccinated with live *Erysipelothrix* vaccine[16,17] prepared from an attenuated strain Koganei 65-0.15[18] of serovar 2 developed localized urticarial lesions at the site of intradermal exposure with serovars 8, 9, 10, or 20. There was a similarity of protection pattern in swine vaccinated with the live-organism vaccine[16,17] to that in swine vaccinated with the adsorbate bacterin,[14,15] and a specific lack of immunity induced by the live *Erysipelothrix* vaccine to certain strains of *E rhusiopathiae* in swine and mice. Seemingly, immunity induced by live-organism vaccine may be caused by specific antigenic stimulation, rather than nonspecific cellular response.

The soluble immunizing antigen(s)[19,20] in whole culture erysipelas bacterins have been described as glycolipoprotein complexes.[21] Most of the antigen is found in the culture filtrate (CF).[22] Culture filtrate of strain Koganei 65-0.15 was effective for protection of mice and swine from challenge exposure with a virulent strain of serovar 1a.[a] A cross-protective effect of CF has been demonstrated against *E rhusiopathiae* strains of serovars A and B (presently serovars 1 and 2) in mice.[21] The purpose of the present report was to determine whether CF prepared from broth culture of an attenuated strain of *E rhusiopathiae* induced cross protection in swine and mice similar to that induced by live-organism vaccine or adsorbate bacterin.

Materials and Methods

Bacterial strains—Strain Koganei 65-0.15[18] (serovar 2) of acriflavine-fast attenuated *E rhusiopathiae* was used for the preparation of CF. Twenty strains of *E rhusiopathiae* representing serovars 1 to 21 and type N (Table 1) were used for challenge exposure of mice and swine. Most of these strains originated from swine; however, 1 strain (Goda) of serovar 8 was the only strain pathogenic for swine among our collections of strains originating from birds and fish. For the determination of LD_{50},[23] mortality of mice was recorded 14 days after subcutaneous (SC) inoculation with 0.1 ml of serial 10-fold dilutions of 24-hour beef infusion broth (BIB) culture of each strain. Two pigs were inoculated intradermally with 0.1 ml of the broth culture of each strain. Each inoculum contained about 10^6 or 10^7 viable bacteria. The strain that induced a local urticarial lesion (≥ 20 mm diameter) or clinical signs of generalized erysipelas in swine within 14 days after inoculation, was recorded as pathogenic for swine.

Preparation of CF vaccine—Frozen cultures of strain Koganei 65-0.15, which had been prepared from the lyophilized stock, were thawed and cultured in BIB (pH 7.6) at 37 C for 24 hours. Beef infusion broth (3 L) containing 0.1% Tween 80[b] was inoc-

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[b] Wako Pure Chemical Industries Ltd, Osaka, Japan.

TABLE 1—Cross-protective effect of culture filtrate in mice to *Erysipelothrix rhusiopathiae* strains of various serovars

Challenge inoculum					No. of susceptible*/ 10 challenge exposed	
Serovar	Strain	Origin	LD_{50} (CFU)	Dose ($\times 10^3$ CFU)	Vaccinated	Controls
1a	Fujisawa†	Swine (septicemia)	4.38	6.04	2	10
1b	H-12†	Swine (endocarditis)	1.35	6.80	0	10
2	Saitama-1‡	Swine (urticaria)	2.70	7.54	0	10
4	2229§	Swine (spleen)	≤ 5.00	1.73	4	10
5	Pécs 67†	Swine (tonsil)	≤ 3.79	1.21	5	10
6	S-17‡	Swine (arthritis)	1.00	4.77	5	10
7	T-334‡	Swine (tonsil)	7.93	6.60	5	10
8	Goda†	Bird	14.00	38.70	0	10
8	911‡	Swine (lymphadenitis)	1.48	5.50	5	10
9	14B§	Swine pen soil	≤ 3.47	2.01	6	10
10	2179§	Swine (spleen)	0.79	1.59	8	10
11	IV 12/8†	Swine (tonsil)	2.34	8.40	3	10
12	Pécs 9†	Swine (tonsil)	22.00	61.00	2	10
15	Pécs 3597†	Swine (tonsil)	4.60	6.70	3	10
16	T-184‡	Swine (tonsil)	0.63	2.26	2	10
18	715§	Swine (spleen)	26.20	85.00	8	10
19	2017§	Swine (spleen)	22.00	50.00	7	10
20	2553§	Swine (spleen)	3.57	6.70	10	10
21	Bâno 36‖	Sheep dip	22.00	59.00	2	10
N	S-12‡	Swine (lymphadenitis)	4.26	8.50	0	10

* Susceptible = dead within 14 days after challenge exposure. † Obtained from Dr. K. Hashimoto, National Institute of Animal Health, Japan. ‡ Obtained from National Veterinary Assay Laboratory, Japan. § Obtained from Dr. R. L. Wood, National Animal Disease Center, Ames, Iowa. ‖ Obtained from Dr. V. Nørrung, State Veterinary Serum Laboratory, Copenhagen, Denmark.
CFU = colony-forming units.

ulated with 30 ml of the subculture and was incubated at 37 C for 24 hours. The number of viable bacteria in the culture was 1.6×10^9 colony-forming units (CFU)/ml. After killing the bacteria by addition of formalin to 0.5% (v/v), the culture was centrifuged at 15,000 × g for 20 minutes at 4 C. The supernatant fluid was passed through a 0.2-μm membrane filter[c] and was designated as CF. The CF was concentrated to 10% of its initial volume by ultrafiltration, using semipermeable membrane.[d] For inoculation, concentrated CF was emulsified in an equal volume of light mineral oil containing a 3% emulsifier,[e] and was used as CF vaccine.

Growth agglutination (GA) test—To determine the agglutinating antibody titer of porcine serum, blood samples were collected from the cranial vena cava of swine before vaccination and at challenge exposure. The GA test[24] was conducted, and titers were expressed as the reciprocal of the number with the highest dilution of serum that had agglutination.

Animals—Six hundred 4-week-old outbred female mice[f] of the ddY strain and eighty 2- to 3-month-old female or castrated male Yorkshire swine[g] were used. Swine were conventionally farrowed and raised in confinement. Swine had GA titers of ≤ 4.

Vaccination and challenge exposure—Mice were inoculated SC at 4 sites in the flank with 0.5 ml of CF vaccine and were randomly allotted into 20 groups of 10 on postvaccination day (PVD) 21. Nonvaccinated control mice were also allotted to 20 groups of 10. Each group was challenge exposed SC in the right inner thigh with 0.1 ml of diluted 24-hour BIB culture (approx 100 LD_{50}) of 1 of 20 strains belonging to 18 serovars and type N (Table 1). The mice were observed each day for 14 days after challenge exposure, and responses were determined by the quantal (live-dead) method.

Swine were inoculated SC twice with 2 and 3 ml of CF vaccine, 3 weeks apart, in the cervical area just caudal to the ear, and were randomly allotted to 20 groups of 2 on the 14th day after the last inoculation of the CF vaccine (PVD 35). Nonvaccinated control swine were also allotted to 20 groups of 2. Each group was challenge exposed intradermally in the flank with 0.1 ml (10^6 or 10^7 CFU) of the BIB culture of 1 of 20 strains. Clinical signs (urticarial lesion, depression, anorexia, difficulty in standing, or death) in swine were observed each day for 14 days after challenge exposure.

[c] Sartorius, Göttingen, Federal Republic of Germany.
[d] Diaflomembrane type PM 10, Amicon Corp, Lexington, Mass.
[e] Arlacel A, Atlas Powder Co, Wilmington, Del.
[f] Shizuoka Agric Coop Assoc Laboratory Animals, Hamamatsu, Japan.
[g] Minano Agric Coop Assoc Laboratory Animals, Saitama, Japan.

Results

Cross protection in vaccinated mice—Control mice died after challenge exposure with all strains. Vaccinated mice survived after challenge exposure with each strain of serovars 1b, 2, 8 (strain Goda), and type N, but mortality occurred in vaccinated mice as follows: 20% to 30% mortality in mice challenge exposed to strains of serovars 1a, 11, 12, 15, 16, or 21; 40% to 50% mortality in mice challenge exposed to serovars 4, 5, 6, 7, or 8 (strain 911); and 60% to 80% mortality in mice challenge exposed to serovars 9, 10, 18, or 19. All vaccinated mice died after challenge exposure with strain 2553 (serovar 20, Table 1).

Cross protection in vaccinated swine—At challenge exposure, the GA titer of serum of vaccinated swine was ≥ 128, whereas nonvaccinated swine retained the titer ≤ 8 (Table 2).

In control groups, 1 of 2 swine challenge exposed to serovar 1a (strain Fujisawa) died in acute phase 4 days after inoculation and another had generalized urticarial lesions with profound depression and anorexia for several days. Swine challenge exposed to serovar 2 (strain Saitama-1) had clinical signs similar to those exposed to serovar 1a; swine challenge exposed to serovar 1b (strain H-12) had somewhat weaker clinical signs than did those challenge exposed to serovars 1a or 2, and swine challenge exposed to serovar 9 (strain 14B) or serovar 10 (strain 2179) developed mild, generalized infection indicated by the presence of 3 to 7 metastatic urticarial lesions for 2

TABLE 2—Clinical responses of swine vaccinated with culture filtrate and nonvaccinated control swine to challenge exposure with *Erysipelothrix rhusiopathiae* strains of various serovars

		Vaccinated				Nonvaccinated			
				Response after challenge exposure				Response after challenge exposure	
Serovar	Strain	Swine No.	GA titer	Erythema	Systemic	Swine No.	GA titer	Erythema*	Systemic
1a	Fujisawa	57	4,096	–	–	56	8	Generalized	+ +
		64	512	–	–	59	4	Generalized	Died
1b	H-12	54	1,024	–	–	55	8	Generalized	–
		61	512	–	–	65	8	Generalized	–
2	Saitama-1	62	1,024	–	–	63	8	Generalized	+ +
		67	2,048	–	–	68	8	Generalized	+
4	2229	69	2,048	–	–	73	4	5 × 6	–
		72	4,096	–	–	79	8	5 × 6	–
5	Pécs 67	74	1,024	–	–	76	4	8 × 11	–
		75	2,048	–	–	77	8	5 × 7	–
6	S-17	24	4,096	–	–	26	8	9 × 10	–
		25	8,192	–	–	27	4	15 × 16	–
7	T-334	76	512	–	–	78	4	10 × 14	–
		77	512	–	–	79	8	10 × 12	–
8	Goda	83	2,048	–	–	85	8	12 × 12	–
		84	4,096	–	–	86	4	12 × 14	–
8	911	28	2,048	–	–	30	4	10 × 13	–
		29	2,048	–	–	31	4	16 × 17	–
9	14B	78	8,192	–	–	80	8	Generalized	–
		81	16,384	–	–	82	8	Generalized	–
10	2179	87	1,024	–	–	89	8	Generalized	–
		88	4,096	–	–	98	8	16 × 18	+
11	IV 12/8	99	1,024	–	–	101	4	17 × 19	–
		100	8,192	–	–	102	4	17 × 17	–
12	Pécs 9	103	4,096	–	–	105	8	9 × 9	–
		104	2,048	–	–	106	4	4 × 6	–
15	Pécs 3597	32	128	–	–	34	4	3.5 × 4.5	–
		33	1,024	–	–	35	4	3 × 3	–
16	T-184	36	512	–	–	38	8	13 × 15	–
		37	512	–	–	39	4	10 × 12	–
18	715	108	256	–	–	110	8	11 × 12	–
		109	256	–	–	111	8	13 × 14	–
19	2017	14	1,024	–	–	16	4	10 × 12	–
		15	512	–	–	17	8	10 × 10	–
20	2553	5	1,024	–	–	7	4	14 × 16	–
		6	512	9 × 10*	–	8	4	10 × 10	–
21	Bâno 36	9	512	–	–	11	4	14 × 14	–
		10	4,096	–	–	13	< 4	4.5 × 5	–
N	S-12	49	1,024	–	–	47	4	10 × 15	–
		50	4,096	–	–	48	4	12 × 16	–

* Maximum size (cm) of erythema at the skin injection site, which was observed for 2 weeks after challenge exposure. + + = Depression, anorexia, and difficulty in standing. + = Temporary depression and anorexia. – = No response. GA = Growth agglutination.

days after exposure. Nonvaccinated swine challenge exposed to the 15 other strains developed local urticarial lesions at the site of intradermal exposure, but had no evidence of generalized infection.

Of 2 vaccinated swine challenge exposed to serovar 20 (strain 2553), 1 developed only a local urticarial lesion. None of the vaccinated swine challenge exposed to 19 other strains belonging to serovars 1a, 1b, 2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 15, 16, 18, 19, and 21 and type N developed clinical signs of acute erysipelas.

Discussion

Specificity in immunity to challenge exposure with various serovars of *E rhusiopathiae* generally does not occur in swine inoculated with the CF vaccine prepared from attenuated strain Koganei 65-0.15. This observation is based on the apparently complete protection induced in vaccinated swine against challenge exposure with most serovars of pathogenic strains. Therefore, the immunizing antigen in the CF was considered to be cross protective.

In the present experiment, CF vaccine induced a complete protection in swine against challenge exposure with the same serovars 9 (strain 14B) or 10 (strain 2179) as previously used,[14,15] although protection rate in mice was relatively low. The standard adsorbate bacterin[14,15] contained 50% of culture supernatant in addition to bacterial cells. The major difference between those 2 vaccines is of adjuvant, which are aluminum hydroxide gel or light mineral oil with mannide monooleate. Therefore, differences of susceptibility of vaccinated swine to these 2 specific strains are probably caused by quantitative dissimilarities of antigenic stimulation between the 2 vaccines.

In the protection test of mice, CF vaccine induced relatively low protection against strains of serovars 9 (40%), 10 (20%), 18 (20%), or 19 (30%) and no immunity against serovar 20 (strain 2553) in comparison with the previous results[16,17] obtained by live-organism vaccine. This difference may also be attributed to a difference in the degree of antigenic stimulation between CF vaccine and live-organism vaccine. Attenuated *E rhusiopathiae*, strain Koganei 65-0.15, grows extensively in the systemic or-

gans for more than 20 days[h] and causes arthritis in inoculated mice.[18,h] Therefore, vaccinated mice are hyperimmunized by long-lasting systemic infection with the attenuated strain, whereas the antigenic stimulation by a single injection of CF vaccine is limited. However, vaccinated swine did not exhibit clinical signs of acute erysipelas against strains of serovars 9, 10, 18, or 19. Of 2 vaccinated swine, 1 developed only a local urticarial lesion against challenge exposure with serovar 20 (strain 2553). Seemingly, these strains are partially distinct from strain Koganei 65-0.15 (serovar 2) used for the preparation of the CF vaccine, and differences in susceptibility of vaccinated mice and swine to the various strains are probably caused by quantitative (rather than qualitative) antigenic dissimilarities between the vaccine strain and certain challenge strains.

Mice vaccinated with live-organism vaccine prepared from the attenuated strain used in the present study did not die after challenge exposure to *E rhusiopathiae* strains of 20 serovars or one N type, but 30% mortality occurred in vaccinated mice challenge exposed to only serovar 20 (strain 2553).[16,17] In the swine protection test, 1 of 2 vaccinated swine challenge exposed to each of serovars 8 (strain 911) or 20, and 2 of 4 vaccinated swine challenge exposed to each of serovars 9 (strain 14B) or 10 (strain 2179) developed localized urticarial lesions at the site of intradermal exposure, but vaccinated swine challenge exposed to serovars 1a, 1b, 2, 4, 5, 6, 7, 11, 12, 15, 16, 18, 19, or 21 or type N did not have clinical signs of acute erysipelas.[16,17] The immunity stimulated by live cells of attenuated *E rhusiopathiae* is reportedly cell mediated.[25] Mice hyperimmunized against *E rhusiopathiae* had a notable cross protection on challenge exposure with virulent *Listeria monocytogenes*.[26] However, in the present experiment, CF vaccine induced a cross-protection pattern in mice and swine similar to that induced by live-organism vaccine in previous experiments.[16,17] The cross protectivity with CF vaccine in swine was stronger than it was with the live-organism vaccine. Seemingly, cross-protective immunity induced by live-organism vaccine may be caused by specific antigenic stimulation, rather than nonspecific cellular response. Common protective antigen, which is found in CF as a soluble immunizing factor, released from the attenuated live cells by their autolysis as a result of continuous multiplication in the skin injection site of swine,[27] may induce the production of cross-protective antibody. Further investigation on the protective effect of anti-CF serum would be helpful to confirm the important role of CF in cross protection.

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The Influence of Preparturient Intramammary Vaccination on Immunoglobulin Levels in Bovine Mammary Secretions

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Summary. Immunoglobulin concentrations were measured in colostrum and milk from individual mammary glands of three cows. Two of the glands of each cow had been vaccinated with a live, formalinized, *E. coli* vaccine. No major differences were found between immunoglobulin concentrations in colostrum from vaccinated and non-vaccinated glands. However, markedly higher concentrations of all immunoglobulins were found in mammary secretions from the vaccinated glands taken 2 and 3 days after calving. IgA concentrations were significantly higher in milk from vaccinated glands than in milk from non-vaccinated glands from day 2 to day 28.

INTRODUCTION

The first suggestion that the mammary gland could be stimulated to produce antibodies was made by Smith, Orcutt and Little (1923). This was based on the observation that cows with active Brucella infections of the mammary gland, and cows vaccinated in the mammary gland with a killed Brucella vaccine, had higher antibody titres in the milk than did cows vaccinated parenterally or cows with non-infected glands.

Mitchell, Walker and Bannister (1953; 1954) recorded the secretion of high levels of neutralizing antibodies to viruses which had been instilled into the mammary glands of cattle. Antibodies were demonstrated later in milk from the non-vaccinated glands and blood serum than in milk from the vaccinated gland. Wilson (1972) demonstrated that cows vaccinated in the mammary gland with *Escherichia coli* antigens responded by secreting milk which contained significantly higher antibody titres than did milk from the non-vaccinated glands.

Enhanced antibody secretion from the mammary gland following local vaccination has been shown in goats (Mitchell, Guerin and Robillard, 1969; Pasieka, Guerin and Mitchell, 1970), sheep (Lascelles, Outteridge and Mackenzie, 1966; McDowell and Lascelles, 1969) and pigs (Wilson, Svendsen and Brown, 1972).

The ability of the bovine and ovine mammary gland selectively to concentrate IgG1 from serum immediately prior to parturition has been recognized (Murphy, Aulund, Osebold and Carroll, 1964; Pierce and Feinstein, 1965; Aalund, 1968). Even though

the absolute immunoglobulin levels are much lower, the electrophoretically fast IgG continues to be selectively transported during lactation in sheep (Mackenzie and Lascelles, 1968) and cattle (Dixon, Weigle and Vasquez, 1961).

The concentration of immunoglobulins in bovine milk and colostrum have been measured by Mach and Pahud (1970), Klaus, Bennett and Jones (1969) and Butler (1971). The purpose of this study was to measure the concentration of the immunoglobulins in bovine colostrum and milk from vaccinated and non-vaccinated mammary glands.

MATERIALS AND METHODS

Cows

Three 18-month-old heifers of the Jersey breed were used. At the start of the experiment it was estimated that they were approximately 1 month from calving.

Vaccine

A tryptic soy broth culture containing $7–8 \times 10^8$ viable bacteria/ml was incubated for 15 hours at 37° in the presence of 0·04 per cent v/v of formalin. The formalinized cultures contained approximately 7×10^6 viable bacteria/ml.

Vaccination

Vaccine was introduced into the teat cistern of the right front (RF) and left hind LH mammary gland via the teat canal.

Heifer 2Z was vaccinated with an antigen prepared from *E. coli* serotype 08: K87; 88a, b; H19. (P307 strain): 4 ml and 6 ml were inoculated into each gland 15 and 5 days before calving respectively.

Heifer W3 was vaccinated with an antigen prepared from *E. coli* serotype 0149:K91; 88a, c; H10 (A1 strain): 4, 6 and 8 ml were inoculated into each gland, 26, 16 and 6 days before calving respectively.

Heifer 3Z was vaccinated with 4, 6 and 8 ml into each gland 28, 18, 8 and 1 days before calving, respectively. The RF gland was inoculated with P307 and the LH gland with A1 vaccines.

Whey

Colostrum or milk was taken from each mammary gland on the day of parturition and on days 2, 3, 7, 14 and 28 post-parturition. These samples are referred to as d1, d2, d3, d7, d14 and d28, respectively.

Whey was prepared from the samples by centrifugation at 44,000 *g* for 2 hours (Bohren and Wenner, 1961) and stored in 2-ml aliquots at −30°.

Antisera

Antisera used in this study were produced in guinea-pigs following the techniques of Binaghi, Orisol and Boussac-Aron (1967). The antigen consisted of immunoprecipitates of the respective immunoglobulins obtained by immunoelectrophoresis (precipitated with guinea-pig antisera having class specificity) and incorporated into Freund's complete adjuvant. Anti-IgA and anti-IgM antisera did not require absorption to render them monospecific as measured by micro gel diffusion, immunoelectrophoresis and radial

immunodiffusion using purified standards of other immunoglobulin classes and a semi-purified secretory component preparation. Most anti-IgG1 and anti-IgG2 antisera failed to react with IgA and IgM but did cross react with the other IgG subclasses. Absorption of these anti-IgG subclass antisera with the other IgG subclasses rendered them mono-specific. (Duncan, Wilkie, Hiestand and Winter, 1972).

Measurement of immunoglobulins

Immunoglobulins were measured in serum and secretions by single radial immuno-diffusion by the method of Fahey and McKelvey (1964) with some modifications. Plates containing appropriate amounts of antisera (1·5 per cent agar in 0·1 M Tris-HCl buffer pH 7·4) were allowed to diffuse for 24 hours at room temperature for measurement of the IgG1, IgG2 and IgA. IgM plates were allowed to diffuse for 48 hours. Plates were stained with 5 per cent acetic acid (Kaufman, 1970), and the ring diameter measured on a microcomparator (Nikon Profile projector). Six standards were included on each plate using purified preparations of IgG1 (colostrum; 6·8S), IgG2 (serum; 6·7S), and IgA secretory; predominately 11S) suspended on 0·1 M Tris-HCl buffer, pH 7·4, containing 1 mM disodium ethylene diamine-tetraacetate (EDTA). Dilutions of bovine serum for which IgM concentrations had been determined by comparison to known concentrations of the purified IgM, were used for IgM standards. The preparation of the immuno-electrophoretic standards has been described by Duncan *et al.* (1972).

The reproducibility of the tests performed is demonstrated by the range and mean coefficients of variation on the six dilutions of immunoglobulin standards from tests performed on the same day; IgG2, 0·10–3·8 per cent ($\bar{X}$ = 1·38); IgG1, 0·34–5·4 per cent ($\bar{X}$ = 2·3); IgA, 0·12–3·8 per cent ($\bar{X}$ = 1·2); IgM 0·60–5·7 per cent ($\bar{X}$ = 2·6).

Statistical analysis

The paired *t*-test (Alder and Roessler, 1967) was used to compare immunoglobulin concentration in whey from vaccinated and non-vaccinated glands. Differences with calculated *t* values greater than at the 0·05 level of probability were considered significant.

RESULTS

The concentration of IgG1, IgG2, IgM and IgA in mammary secretions on days 1 to 28 after calving from immunized and non-immunized mammary glands are given in Table 1. The mean immunoglobulin levels in vaccinated and non-vaccinated gland secretions are shown in Table 2.

IgG1 was found to be the predominant immunoglobulin in colostrum, the levels dropping rapidly to day 7 and more slowly thereafter. Apart from the day 1 sample the mean IgG1 concentration was consistently higher in milk from vaccinated glands, especially on days 2 and 3, statistically significant differences were seen between IgG1 levels on days 2, 3, 7 and 28. IgG1 was still the predominant immunoglobulin in secretions taken 28 days after calving.

IgG2 was from 1/6 to 1/20 the level of IgG1 in samples taken on day 1 and, like IgG1, decreased rapidly to day 7 and more slowly thereafter. Significant differences in IgG2 concentrations between vaccinated and non-vaccinated secretions were found on days 2, 3 and 28.

TABLE 1

IMMUNOGLOBULIN CONCENTRATIONS IN MAMMARY SECRETIONS FROM COWS VACCINATED IN THE MAMMARY GLAND WITH *Escherichia coli* ANTIGENS

Cow No./Ig	Gland	Days after calving: 1	2	3	7	14	28
		(mg/ml)					
2Z/IgM	RH	3·15	0·54	0·11	0·15	0·125	0·03
	RF	3·35	3·40	0·68	0·10	0·105	0·03
	LH	3·2	0·76	0·38	0·14	0·14	0·03
	LF	3·65	0·28	0·125	0·21	0·17	0·04
2Z/IgG1	RH	41·0	8·0	1·89	0·60	0·34	0·24
	RF	35·0	18·0	2·60	0·72	0·44	0·31
	LH	36·0	13·5	2·44	0·64	0·37	0·275
	LF	38·0	3·85	1·71	0·62	0·34	0·22
2Z/IgG2	RH	4·0	0·67	0·18	0·08	0·04	0·03
	RF	4·25	2·25	0·82	0·08	0·055	0·04
	LH	3·70	1·60	0·44	0·10	0·05	0·04
	LF	3·80	0·44	0·19	0·06	0·05	0·03
2Z/IgA	RH	1·35	0·255	0·09	0·08	0·09	0·07
	RF	1·47	1·04	0·32	0·22	0·28	0·22
	LH	1·45	0·81	0·28	0·18	0·24	0·16
	LF	1·65	0·123	0·07	0·07	0·10	0·09
W3/IgM	RH	2·53	0·60	0·25	0·18	0·11	0·08
	RF	2·33	1·26	0·51	0·22	0·08	0·12
	LH	3·06	0·87	0·40	0·26	0·15	0·11
	LF	3·46	0·50	0·32	0·26	0·11	0·10
W3/IgG1	RH	17·7	3·75	1·42	0·50	0·34	0·23
	RF	12·1	4·85	2·23	0·60	0·26	0·29
	LH	21·0	5·50	1·83	0·68	0·41	0·33
	LF	24·5	3·95	1·26	0·60	0·36	0·30
W3/IgG2	RH	0·95	0·22	0·09	0·07	0·04	0·03
	RF	0·86	0·49	0·17	0·07	0·04	0·04
	LH	0·91	0·54	0·15	0·09	0·06	0·05
	LF	1·03	0·28	0·11	0·07	0·05	0·04
W3/IgA	RH	0·98	0·12	0·07	0·05	0	0·05
	RF	1·06	0·68	0·325	0·16	0·12	0·09
	LH	1·02	0·40	0·21	0·15	0·10	0·065
	LF	0·59	0·11	0·06	0·05	0·05	0·05
3Z/IgM	RH	6·1	2·0	0·29	0·13	0·145	0·07
	RF	4·4	3·10	0·46	0·12	0·09	0·05
	LH	4·4	3·70	1·45	0·12	0·08	0·05
	LF	4·2	1·15	0·22	0·13	0·105	0·06
3Z/IgG1	RH	49·0	27·0	2·34	0·85	0·32	0·37
	RF	33·0	33·0	5·7	0·96	0·305	0·43
	LH	25·5	32·5	8·6	1·17	0·77	0·5
	LF	50·0	20·0	2·95	0·80	0·35	0·375
3Z/IgG2	RH	5·75	1·45	0·39	0·09	0·05	0·03
	RF	6·0	3·8	0·76	0·11	0·05	0·05
	LH	6·3	3·4	2·8	0·16	0·09	0·05
	LF	5·5	1·4	0·28	0·08	0·055	0·04
3Z/IgA	RH	2·25	0·43	0·152	0·10	0·08	0·07
	RF	3·5	1·15	0·47	0·195	0·165	0·14
	LH	1·7	1·53	1·27	0·355	0·36	0·27
	LF	1·94	0·31	0·17	0·08	0·06	0·07

Cow 2Z vaccinated in RF and LH with *E. coli* serotype 08 : K87; 88a, b: H19 (P307 strain).
Cow W3 vaccinated in RF and LH glands with *E. coli* serotype 0149 : K91; 88a, c: H10 (A1 strain).
Cow 3Z vaccinated in RF gland with the A1 strain and in the LH gland with the P307 strain of *E. coli*.
RH = right hind; RF = right fore; LH = left hind; LF = left fore.

TABLE 2

MEAN IMMUNOGLOBULIN CONCENTRATIONS IN SECRETIONS FROM VACCINATED AND NON-VACCINATED MAMMARY GLANDS OF COWS

Ig	Treatment	Days after calving: 1	2	3	7	14	28
		(mg/ml)					
IgM	Non-vaccinated	3·85 (1·12)	0·85 (0·56)	0·22 (0·07)	0·18 (0·05)	0·18 (0·0)	0·06 (0·0)
	Vaccinated	3·46 (0·74)	2·18* (1·24)	0·65*(0·37)	0·16 (0·06)	0·11 (0·0)	0·07 (0·0)
IgG1	Non-vaccinated	34·04 (11·37)	11·09 (9·12)	1·93 (0·57)	0·66 (0·12)	0·34 (0·0)	0·29 (0·05)
	Vaccinated	27·10 (8·57)	17·89*(11·43)	3·90*(2·46)	0·79*(0·20)	0·43 (0·16)	0·36* (0·08)
IgG2	Non-vaccinated	3·50 (1·91)	0·74 (0·50)	0·21 (0·10)	0·07 (0·0)	0·05 (0·0)	0·03 (0·0)
	Vaccinated	3·67 (2·18)	2·01 (1·28)	0·86*(0·90)	0·10 (0·0)	0·06 (0·0)	0·045*(0·0)
IgA	Non-vaccinated	1·46 (0·56)	0·22 (0·13)	0·10 (0·04)	0·07 (0·14)	0·06 (0·03)	0·06 (0·01)
	Vaccinated	1·67 (0·77)	0·94* (0·36)	0·48*(0·36)	0·21*(0·68)	0·21*(0·09)	0·16 *(0·07)

Vaccinated cows received *E. coli* antigens in the right front and left hind mammary glands. Figures in parentheses are standard deviations of the mean.

* Significant difference between mean of vaccinated and non-vaccinated.

IgM concentrations were approximately equal to IgG2 levels in cows 2Z and 3Z but in cow W3 were three times the IgG2 levels. As for IgG1 and IgG2, the difference in IgM levels between vaccinated gland secretions were particularly marked on days 2 and 3. The differences on days 2 and 3 were statistically significant.

Day 1 IgA concentrations were lower than any other immunoglobulin. The IgA concentration in secretions from vaccinated glands on days 2, 3, 7, 14 and 28, were significantly greater than the concentrations found in milk from non-vaccinated glands. Absolute levels of IgA in secretions from both vaccinated and non-vaccinated glands were higher than IgG2 in all cows on days 14 and 28 and higher than IgM in cows 2Z and 3Z on day 28.

The ratio of IgG1 and IgG2 in day 1 secretions was approximately 10:1 and was the same in day 28 secretions.

The marked difference in immunoglobulin concentration in the colostral (day 1) samples among cows and among quarters in the same cow (independent of vaccination) should be noted.

DISCUSSION

Preparturient intramammary vaccination has clearly altered the immunoglobulin content of post-parturient bovine mammary secretions. The milk whey from vaccinated glands contained greater concentrations of IgG (IgG1 and IgG2) and IgM on days 2 and 3 and IgA on days 2 to 28. Mechanisms responsible for this could be an increase in vascular permeability, an increase in selective transport from serum, or production of the immunoglobulins in the mammary gland.

Mechanisms responsible for the large differences in immunoglobulin concentrations between samples taken from vaccinated and non-vaccinated glands on days 2 and 3 remain obscure. Since IgM and IgG2 concentrations did not differ so markedly in secretions from vaccinated and non-vaccinated glands taken after day 3 it would suggest that the initial increase was due to either non-selective transport from serum, or enhanced local antibody synthesis of IgM, and IgG2 which was of a much shorter duration than that of IgA. The significant increases in IgA and IgG1 levels associated with vaccination are probably the result of local antibody production in the mammary gland to either the broth diluent or the bacterial antigens. Increased levels of IgA (Lascelles and McDowell, 1970), and greater numbers of IgA synthesizing plasma cells (Lee and Lascelles, 1970), have been demonstrated in antigenically stimulated mammary glands from ewes.

In this study the use of 11S IgA standards to quantify secretory IgA immunoglobulins would tend to give lower readings than may exist if IgA immunoglobulins of a higher sedimentation velocity were present. Monomeric (7S) IgA has not been demonstrated in cattle. (Mach and Pahud, 1970; Vaerman, 1970; Duncan *et al.*, 1972).

Bovine IgG1 is preferentially secreted into colostrum prior to parturition (Murphy *et al.*, 1964; Pierce and Feinstein, 1965; Dixon *et al.*, 1961). The ratio of IgG1:IgG2 in colostrum found in this study was approximately 10:1 and the same ratio existed in day 28 milk. Therefore, if a selective transfer of IgG1 operates on day 1, the same mechanism would appear to operate on day 28. In contrast, an IgG1:IgG2 ratio of 3:1 to 2:1 has been found in nasal secretions and tears from non-lactating heifers (Duncan *et al.*, 1972). It remains to be seen what influence lactation has on IgG transport to other epithelial membranes.

Although marked increases were not found in the concentration of any of the immunoglobulin classes between vaccinated and non-vaccinated gland samples taken on day 1, qualitative differences were found in the form of increased indirect haemagglutinin antibody titres in the samples from vaccinated glands. Bactericidal activity was not found in samples taken from any gland on any day, but inhibition of bacterial multiplication occurred in all samples through day 3 and was demonstrable in vaccinated gland samples to day 28. (Wilson, 1972). Studies to determine in which immunoglobulin class the specific antibody activities reside have not been performed.

The enhanced immunoglobulin production in the vaccinated mammary gland which persisted for up to 28 days after antigen administration could be of practical significance in the prevention of mastitis, or in prophylaxis against enteric infections in newborn animals ingesting milk secreted from such glands. Since secretory immunoglobulin is more resistant to digestion than other immunoglobulins (Brown, Newcomb and Ishizaka, 1970; Stewart, 1971) and has the tendency to adhere to epithelium and resist absorption (South, 1971), the increased levels of immunoglobulin found in milk as a result of local vaccination (and presumably specific antibody in the same class) could be of major significance as far as immune prophylaxis to enteric infections is concerned.

The marked inter-cow variation of absolute immunoglobulin concentrations in day 1 mammary secretions could be of importance in the pathogenesis of hypogammaglobulinaemia of newborn calves since the absolute mass of immunoglobulin ingested (Kruse, 1970) does influence the subsequent serum immunoglobulin levels attained by calves.

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anthelmintic treatment and washing sows prior to farrowing is often recommended as a means of reducing the transmission of nematode infection from sows to their piglets. While washing sows appeared to reduce the prevalence of infection in all classes of growing pigs, the association was only significant for baconers. In this survey, prefarrowing anthelmintic treatment of sows was associated with a lower prevalence of worms but this was not significant (P = 0.1). Measures to prevent the transmission of worm infection from sows to piglets are particularly important since sows have been found to be the most commonly infected class of pig (Mercy *et al* 1989).

Because of the relatively small number of herds involved in some of the management and housing categories and the possibility of confounding between variables, the associations found in this study are not necessarily casual relationships. Further studies would be required to determine the nature of these associations.

The results of this survey have a number of implications for pig producers. Firstly, despite the relatively high level of usage of anthelmintics in pig herds, many were still infected with nematode parasites. In many cases a more appropriate administration of deworming drugs may be required while in others, a better choice of anthelmintic is necessary. Secondly knowledge of the species of worms present in herds would be an advantage to pig producers in selecting the most appropriate anthelmintic to use. Work is required to determine the most appropriate deworming strategy for commercial piggeries given the husbandry practices normally used.

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Protection against enzootic pneumonia of pigs: intraperitoneal inoculation with live LKR strain of *Mycoplasma hyopneumoniae*

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SUMMARY: Pigs obtained from a mycoplasma-free piggery were randomised into 4 groups of 9. Groups 1 and 2 were injected by the intraperitoneal route with liquid culture of the LKR strain of *Mycoplasma hyopneumoniae.* Group 1 was injected once and group 2 twice. Group 3 was made up of pigs inoculated by the intranasal route with the virulent Beaufort strain of *M. hyopneumoniae;* they served as the source of infection for the challenge. Group 4 were uninfected, uninjected controls. Six weeks after the last injection, groups from 1 to 4 were placed in contact.

Seven of the pigs in the 1-dose group and 6 in the 2-dose group were free of lesions at necropsy 6 weeks after challenge. Of the two pigs with lesions in the 1-dose group one had only a small lesion but the other had extensive lesions; it had not shown an antibody response after injection of culture. The lesions in the 3 pigs in the 2-dose group were all small. All 9 control pigs had lesions which varied from medium to large in size. The difference in the incidence of pneumonia between the injected and control groups was significant ($P<0.05$) and the proportion of severely affected pigs in the vaccinated groups was significantly lower ($P>0.01$). There was no difference between those given one dose of vaccine and those receiving 2 doses.

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Introduction

Studies by Etheridge and Lloyd (1982) showed that induced resistance to enzootic pneumonia of pigs could be demonstrated by exposing injected and not injected pigs to artificially infected pigs and comparing the degree of lung infection in the 2 groups. At least 60% of the pigs injected with the LKR strain of *Mycoplasma hyopneumoniae* by the intravenous, intraperitoneal or subcutaneous routes were free of lung lesions when necropsied 5 to 7 weeks after exposure wheras all but one of the 32 uninjected control pigs had lung lesions.

While these studies showed that resistance of pigs to enzootic pneumonia could be induced, the methods used to provoke resistance had 2 disadvantages. The first was that many injected pigs developed arthritis. This was especially marked in the intravenous group where all pigs were affected and most showed polyarthritis. In the intraperitoneal group fewer pigs and fewer joints were affected and in the subcutaneous group only one pig showed arthritis. The second disadvantage was the difficulty in determining whether injected pigs had responded to the organisms. While all those injected intravenously showed positive CF titres, 3 of those injected by the intraperitoneal route and 7 of those by the subcutaneous route did not. It was assumed that, with the latter 2 routes, antigen had not reached an immunology reactive site in every animal.

There was no correlation between a positive CF response and protection. When challenged, most pigs with CF titres

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TABLE 1
Results of exposure of injected and uninjected pigs to pigs with induced enzootic pneumonia

Response to injection					Response to challenge				
Group* number	Mean age at inoculation (days)	Serological response dose 1	dose 2	No response	Necropsy Pneumonia Nil	1 lobe	2 or more lobes	*M hyopneum.* isolated	Mean severity score
1/9	75.6	6 at 11d	n.a.†		6	0	0	2	0.31**
		2 at 18d			1	1	0	0	
								1	
				1			1		
2/9	50.6	4 at 8d			2	1	1	2	0.21**
		3 at 15d			3	0	0	2	
			1 at 4d†		1	0	0	1	
				1		1		1	
controls 4/9	n.a.	n.a.	n.a.	n.a.	0	0			2.4
							9	9	

* groups are described in text
n.a. = not applicable
† response occurred 4 days after second dose
**pneumonia less severe in vaccinates ($p<0.01$)
* fewer vaccinates infected ($p<0.05$)

were protected and some without titres were also protected. Conversely some of those with CF responses were not protected.

If live LKR strain is to be considered as a candidate vaccine it should produce little or no arthritis and a high level of protection. To these ends the intraperitoneal route was chosen for further studies; it gave a better serological response and slightly better protection than the subcutaneous route and produced far less arthritis than the intravenous route.

A series of experiments using the intraperitoneal route and designed to test dose, age at effective injection and effect of injection of sows before farrowing on resistance, were unsatisfactory. Significant differences between injected and uninjected pigs could not be demonstrated even though some injected pigs were obviously resistant to injection (LC Lloyd unpublished data).

The present experiment was designed to determine whether shortcomings in the injection procedure were responsible for the variable results. To this end, animals were prepared, and the injection was made, with special care. In addition, in order to detect whether pigs had not received a dose of vaccine at a reactive site, one group of pigs was injected twice, the second dose being at a time when the first dose should have produced a serological response. If non-reactors reacted to the second injection it could be assumed that the first was ineffective.

Materials and Methods

Organisms and Culture Medium

The *M.hyopneumoniae* strains J and Beaufort and the MH medium were described previously (Etheridge and Lloyd 1982). The strain used as a vaccine was a cloned isolate of LKR (Lloyd and Etheridge 1981) taken from an arthritic joint of a pig that had been inoculated with LKR strain by the intrathoracic route.

Experimental Animals

Pregnant Landrace x Large White sows known to be free of enzootic pneumonia were kept in isolation where they farrowed. Piglets were tattooed in the ear, injected with an iron preparation* and vaccinated against erysipelas† They were weaned when 5 weeks old and separated into 4 groups of nine.

* Dexavin®, Pfizer Agricare Pty Ltd, West Ryde, New South Wales
† Eryvac®, Commonwealth Serum Laboratories, Parkville, Victoria

Inoculation of Culture and Preparation of Donor Pigs

These procedures were described by Lloyd and Etheridge (1981) and Etheridge and Lloyd (1982) respectively.

Necropsy Procedure

This was described by Lloyd and Etheridge (1981). An additional step in assessing whether *M.hyopneumoniae* was present in lungs was that washings of both left and right bronchi and associated bronchioles were recovered and sown into MH medium. The donor pigs were slaughtered at an abattoir; joints could not be inspected. The injected and the control pigs were killed at the Experiment Station where a full examination was done. In assessing the severity of infections, scores (Etheridge and Lloyd 1982) of less than 1 were classified as minor infections whereas pigs with scores greater than 1 were considered to be moderately or severely affected.

Monitoring

All pigs were bled for CF testing before entering the experiment and then each week for the duration of the experiment. In addition, blood was taken for culture from injected pigs each week in the period between inoculation and challenge. One ml of fresh blood was inoculated into 9 ml of MH selective medium.

Pigs were inspected for signs of lameness and, after the challenge began, for signs of respiratory involvement such a coughing and increased respiratory rate.

Experimental Design

At the start of the experiment group 2 was injected by the intraperitoneal route with LKR strain. Three weeks later group 2 and group 1 were injected by the same route with the same strain. Five weeks later group 3 were inoculated by the intranasal route with the Beaufort strain of *M.hyopneumoniae.* A week later the challenge began; group 3, group 4 and groups 1 and 2 were reassembled into 3 groups of 12 pigs; each group contained 3 donor pigs, 3 susceptible controls, 3 injected once and 3 injected twice. This constituted the challenge.

Thirteen days after the challenge began, 3 donor pigs in one group were CF negative and were not coughing. As this suggested that the challenge might not work, 2 of these donors were exchanged with one reacting, coughing donor pig from each of the other 2 groups. Soon after the exchange, those from the first group seroconverted and began to cough. This precautionary manoeuvre did not compromise the design.

Six of the donor pigs were killed 29 days after challenge began and the other 3, 16 days later. Necropsies of the control

and injected pigs began 44 days after the start of the challenge and were completed 22 days later.

Statistical Analysis

The presence or absence of pneumonia is a binomial variable and can be analysed by fitting a generalised linear model (with binomial errors and logit link) using the statistical package GLIM (release 3, Baker and Nelder 1978). This enables the fitting of covariates as well as treatment effects. Thus it is possible to test the effect of such factors as litter or group and covariants, such as the time of necropsy, on the probability that an individual pig contracts pneumonia.

Results

Response to Injection

Eight of the 9 pigs in group 1 developed CF antibody titres at a mean of 12.75 (SE 1.1) days after injection (Table). Four pigs showed mycoplasmaemia, 3 on only one day at 11, 18 and 39 days post injection and one at 4 consecutive weekly bleedings commencing at day 11 post inoculation. None of the pigs was lame. The dose of organisms in this inoculum was at least 5.0×10^7 and probably greater.

After the first injection 7 of the 9 group 2 pigs developed CF antibody titres at a mean of 11 d (SE 1.4) after injection (Table). One of the CF negative pigs developed a CF response 4 d after the second injection but the other remained negative until 35 d after challenge. Two pigs showed mycoplasmaemia, both on one day only, one at 8 d after the first injection and the other 18 d after the second injection. None was lame. The dose of organisms in the first inoculum was 2.6×10^{10} and in the second it was the same as that used for the one dose group.

Challenge of Resistance

Group 3

All pigs showed lesions of enzootic pneumonia at necropsy 36 to 52 d after intranasal inoculation and *M.hyopneumoniae* was isolated from lung lesions of 8 and from washings of bronchi of 8. The average score for the lesions was 2.9 (SE 0.3). All pigs showed a serological response beginning at a mean of 17.2 d (SE 2.2) after inoculation. The histopathology was typical of that for enzootic pneumonia. The joints were not examined.

Group 4

All pigs showed lung lesions at necropsy 44 to 59 d after exposure and *M.myopneumoniae* was isolated from lesions in all 9 pigs and from washings of the bronchi in 4 (Table). The mean score was 2.4 (SE 0.4). The histopathology was typical of that for enzootic pneumonia. All 9 pigs developed a serological response. The mean time from exposure of infected pigs to the first positive CF response was 22.7 d (SE 1.7).

The long bone joints were examined in all pigs but none was arthritic.

Response to Challenge in Vaccinated Pigs

Group 1

When necropsies were carried out, 52 to 64 d after exposure, 7 of the 9 pigs were free of lesions (Table). One pig showed a small lesion, score 0.37 and another a large lesion, score 2.47. The latter was in the pig that did not give a CF response to the injection of culture. The lesions in both pigs were indistinguishable histologically from those of enzootic pneumonia. *M.hyopneumoniae* was isolated from the lung tissue of the pig with the larger lesion and from the lung of another pig that did not have a lesion. The washings from the bronchi of yet another pig were positive for this organism. Eight of the 9 showed an increase in CF antibody about 26 d after challenge.

Even though lameness was not apparent after injection one pig had arthritis in 3 joints; *M.hyopneumoniae* was isolated from 2 of these. It was also isolated from a joint in another pig but arthritis was not confirmed when the joint capsule was examined histologically.

Group 2

When necropsies were carried out 50 to 66 d after exposure, 6 of the 9 pigs were free of lesions (Table). A single lesion, score less than 1, was seen in each of the 3 other pigs. *M.hyopneumoniae* was isolated from the washings of a bronchus in 2 of the 3 pigs. It was also isolated from the lungs of 2 pigs without lung lesions, from lung washings of 2 pigs without lung lesions, from an excess of peritoneal fluid in one and from the pericardial fluid of another. The latter was increased in amount and slightly cloudy. The inner surface of the pericardial sac showed patchy and slight thickening.

The CF antibody response was greater in 6, unchanged in 2 and reduced in one. The maximum response was at 26 d which was the same as that of the 1-dose group.

Two pigs showed arthritis in one joint each, which was confirmed histologically, and *M.hyopneumoniae* was isolated from both. As in Group 1 they were not lame after injection.

Statistical Analysis

This indicated that:

- The proportion of pigs infected was significantly lower ($P<0.05$) in the injected groups than in the control group and also the proportion of pigs severely infected was significantly lower ($P<0.01$) in the injected groups than in the control group.
- There was no significant difference between the 1-dose and 2-dose groups in the proportion of pigs infected and also in the proportion of pigs severely infected. There were no effects due to the litter of origin or the group to which the pig was assigned.
- Although the mean interval from challenge to necropsy was lower in the control group than in the injected groups, the probability that a pig contracted pneumonia was not altered by the interval to necropsy.

Discussion

In this experiment intraperitoneal injection of live LKR strain of *M.hyopneumoniae* conferred a considerable degree of protection against infection transmitted from artificially infected pigs. It confirms the results of the previous study (Etheridge and Lloyd 1982) and raises the question as to whether live organisms can be used to protect pigs against enzootic pneumonia in commercial situations. Experiment have been reported in which either killed *M.hyopneumoniae* or extracts of it, used as inocula, have induced protection (Lam and Switzer 1971; Goodwin and Whittlestone 1973; Durisic *et al* 1975; Slavik *et al* 1979; Ross *et al* 1984; Kishima *et al* 1985). However none of these has been developed commercially. In one case this was because the procedure was unacceptable for animal welfare reasons but the others may have been due to cost. The present experiment suggests live vaccine may be a satisfactory alternative.

While there was no difference in the degree of resistance in the 1 and 2-dose groups there was evidence that 2 doses were desirable. In the 1-dose group one pig that did not develop a CF response had lesions at necropsy of a size that indicated it had not been protected by injection. In the 2-dose group one pig did not show a response to the first injection but did to the second and at necropsy it was free of lesions which suggests the second dose provoked protection. Thus in some pigs the inoculum did not reach an appropriate site; by giving a second dose the probability that all pigs will be protected, is increased.

Most pigs injected with culture developed a positive CF titre. This was used as an indicator that *M.hyopneumoniae* antigen had made contact with the immune system. It was not taken to mean that the reacting pigs were resistant to infection. In the early stages of this work it was not appreciated that injection by the intraperitoneal route could produce mark-

edly diffrent degrees of resistance. In several other protection experiments (Lloyd and Etherirge, unpublished data) this variation was clearly responsible for the failure to show differences between vaccinated and unvaccinated pigs. In the experiment described here, where injections were made with special care, at lease 2 of the 27 injections did not reach an appropriate site and failed to indue an immune response. We have persevered with intraperitoneal inoculation for the reasons given earlier; it led to less arthritis than intravenous injection and it had produced more CF reactors than did subcutaneous inoculation.

Isolation of *M.hyopneumoniae* from, and suggestions of abnormality in, the peritoneum and the pericardium in separate pigs has not been seen previously in our experiments. Their significance is not known.

Clinical arthritis was not seen in either group of injected pigs, but at necropsy 1 pig in the 1-dose group and 2 in the 2-dose group had joint lesions. Two of the three, one in each group, showed mycoplasmaemia. Altogether 6 injected pigs developed mycoplasmaemia. While this indicates there is some relationship between mycoplasmaemia and arthritis, it is not sufficiently strong to be demonstrable statistically.

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Cerebellar abiotrophy in crossbred cattle

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SUMMARY: Cerebellar abiotrophy affected 9 of 74 calves sired by a Poll Hereford bull over 2 successive calving seasons. The disease was characterised by episodes of recumbency and ataxia, with hypermetria and wide base stance. Clinical signs commenced between birth and 8 months of age. Two calves which were affected first at 8 months of age recovered clinically 9 months later. Histological lesions were found in the cerebellar cortex of 7 calves and consisted of segmental degeneration and loss of Purkinje cells, and axonal swellings. The clinical signs and pathological findings were consistent with bovine familial convulsions and ataxia, which has not been described previously in Australia. The clinical signs were not attributable to the lesions observed in the cerebellum and an underlying electrophysiological abnormality is proposed. The aetiology of the condition is probably genetic and appears to have a multifactorial basis.

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Introduction

Cerebellar diseases are among the most common neurological disorders of domestic animals. They have been reported frequently in cattle and have been classified according to the age at onset of clinical signs and the type of pathology (Cho and Leipold 1977; DeLahunta 1983). Most of the diseases are thought to have a genetic basis, but bovine virus diarrhoea-mucosal disease (BVD-MD) virus is recognised as an infectious cause. Barlow *et al* (1968) described the condition of bovine familial convulsions and ataxia affecting Angus cattle in which cerebellar cortical atrophy (abiotrophy) was found microscopically. An identical clinical and pathological syndrome was reported later in a Charolais calf (Cho and Leipold 1978).

In this report we describe an outbreak of cerebellar abiotrophy that affected the progeny of cows of several breeds mated to a Poll Hereford bull. The condition was consistent with familial convulsions and ataxia, which has not been reported previously in Australia. Epidemiological evidence suggested that the syndrome was heritable.

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History and Clinical Signs

Neurological disease was observed in calves born in autumn 1984 and 1985 on a 110 ha farm near Tumbarumba, in southern New South Wales. The farm consisted of undulating improved pasture and a recently cleared bush paddock adjacent to timbered hills. No recognised poisonous plants or chemicals were found on the farm, although the improved pasture contained perennial ryegrass *(Lolium perenne)*. During 1984 the herd consisted of 37 cows (Murray Grey, Murray Grey X Hereford, Angus X Hereford and Angus X Jersey) which had been mated to a newly purchased, 1-year-old, Poll Hereford bull obtained from a registered stud. In addition, in 1984, there were 10 heifers of mixed breed which had been mated to an Angus bull.

A total of 9 affected and 65 normal calves were produced from the Poll Hereford matings in the 1984 and 1985 calvings. Calves of both sexes were affected. Six were clinically affected at or within 7 days of birth and 3 developed neurological signs at 3 to 8 months of age. Affected calves were produced by cows from all the breed types present. One cow, an Angus X Jersey, produced an affected calf in both calving seasons. All calves produced by the Angus bull were normal. Because of

Protection of Foals Against Experimental *Rhodococcus equi* Pneumonia by Oral Immunization

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ABSTRACT

Two groups of three one to three week old foals were immunized orally on four occasions over five weeks with two strains of *Rhodococcus equi*, a clinical isolate from a pneumonic foal and a laboratory passaged Congo red negative variant of this strain. Three nonimmunized foals of similar age acted as controls. Three weeks after the last immunization, all foals were challenged on five occasions over seven days by aerosol infection with about 10^{10} of the pneumonic foal isolate on each occasion. Control foals became seriously ill and were euthanized. Immunization with either strain protected foals equally against the challenge, and resulted in rapid lung clearance. Oral immunization can thus protect foals against severe challenge with *R. equi*. The proteins associated with Congo red colony staining appear not to be involved in protective immunity.

Key words: *Rhodococcus equi*, foals, immunization.

RÉSUMÉ

Cette expérience portait sur deux groupes de trois poulains âgés d'une à trois semaines que les auteurs immunisèrent, par la voie buccale, à quatre occasions en cinq semaines, avec deux souches de *Rhodococcus equi* dont l'une provenait d'un poulain atteint d'une pneumonie clinique et l'autre, d'une variante de cette souche soumise à des passages au laboratoire et négative à la coloration au rouge Congo. Trois poulains non vaccinés et d'un âge équivalent servirent de témoins. Trois semaines après la dernière immunisation, tous les poulains subirent cinq infections de défi, en sept jours, au moyen d'aérosols qui, à chaque occasion, contenaient environ 10^{10} bactéries de la souche isolée du poulain atteint de la pneumonie clinique. Les témoins devinrent gravement malades et on en effectua l'euthanasie. L'immunisation avec l'une ou l'autre des souches précitées protégea également les poulains contre les infections de défi et favorisa une clairance pulmonaire rapide. L'immunisation orale peut par conséquent protéger les poulains contre une forte infection de défi avec *R. equi*. Les protéines associées à la coloration au rouge Congo ne semblent pas impliquées dans l'immunité protectrice.

Mots clés: *Rhodococcus equi*, poulains, immunisation.

INTRODUCTION

Rhodococcus equi is an important cause of suppurative bronchopneumonia of foals in certain parts of the world. In Ontario it was reported to cause 10% of deaths of foals under six months of age (1).

No effective immunoprophylactic measures have been described and many aspects of immunity to *R. equi* pneumonia in foals remain unclear. While *R. equi* appears to behave as a typical facultative intracellular parasite (2,3), in which protection would be expected to be primarily by cell-mediated immune mechanisms, recent studies (4,5) suggest that humoral immunity may also be important in protection against the disease. The peak incidence of the disease, in six to eight week old foals, coincides with the time when maternally derived antibody is waning. Prescott and others (6) had evidence that oral challenge of foals with *R. equi* led to immunization. Recently Takai and others (5) reported that *R. equi* administered orally to foals resulted in significant increase in specific IgG levels in serum.

The purpose of the present study was to determine whether oral immunization with live *R. equi* would protect against experimental challenge, and to compare the protection afforded by two strains. One strain was a low-passage isolate from the lung of a foal with pneumonia, and the other was a variant of this strain which differed only in the absence of two proteins, a dominant 17.5 kd and a minor 15 kd protein. This strain, unlike its parent, did not produce a red colony when grown on media containing Congo red dye. In other facultative intracellular pathogens Congo red staining has been associated with virulence (7,8).

MATERIALS AND METHODS

FOALS

Three groups of three one to three week old crossbred pony foals were kept, with their dams, on separate pastures, which had been used for horses for several years. Group CR+ (mean age 1.5 weeks) were immunized orally with a live Congo red positive variant of *R. equi* and group CR- (mean age 2.8 weeks) were immunized orally with a live Congo red negative

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variant of the same strain *R. equi*. Group C foals (mean age 1.6 weeks) were nonimmunized controls.

BACTERIA

Rhodococcus equi strain 2523-85 was isolated from the lung of a pneumonic foal and passaged three times *in vitro* before being stored at -70°C. Colonies staining red on Congo red containing media (8) are described here as CR+. A Congo red negative variant of this strain, CR-, was obtained after passage *in vitro* 100 times. This strain was also stored at -70°C. Bacteria were grown in nutrient broth for 48 h at 37°C.

IMMUNIZATION PROCEDURES

Group CR+ foals were given 100 mL phosphate buffered saline, pH 7.2, containing 10^9-10^{10} CR+ *R. equi* and group CR- foals the same number of CR- bacteria, on four occasions. Administration of this dose by stomach tube was at the time of group separation, and again two, three and four weeks later. Following administration of bacteria the stomach tube was flushed with water to decrease the chance of pharyngeal contamination when the tube was withdrawn. Foals were weaned three weeks after the last oral immunization and placed separately in their groups in isolation facilities for experimental challenge with *R. equi*.

EXPERIMENTAL CHALLENGE

All foals in each group were challenged by aerosol with the CR+ strain, using a method based on that described by Martens and others (9). The aerosol was generated by an ultrasonic nebulizer with a disposable drug vial (Hospal Medical Co., Montreal, Quebec). Twenty milliliters of a suspension of 6.6×10^8 *R. equi* were placed in the disposable drug vial which was attached to an inhalation mask. Foals were challenged for 15 min. During this time about 18 mL of the suspension was aerosolized, the majority of which (1.2×10^{10} colony forming units) was judged to have been inhaled. This dose was administered on days 0, 1, 2, 6 and 7 of the study.

CLINICAL AND POSTMORTEM EXAMINATION

Blood samples were taken before each vaccination and after challenge for hematological examination. Following challenge, foals were examined daily. Temperatures, pulse and respiratory rates were recorded. Foals in group C were euthanized ten days after initial aerosol challenge, together with one foal from each of groups CR+ and CR-. The remaining foals were euthanized on day 14. Quantitative bacteriological examination was done at the same four predetermined sites in the lungs of all foals. Sections of lung and intestine were fixed in 10% buffered formalin immediately after death and histological sections examined using routine histopathological procedures. Sections were stained with hematoxylin and eosin (H & E) and Brown and Brenn's modification of the Gram stain.

RESULTS

Foals in each of the groups remained healthy and no changes in total or differential white blood cell count occurred during the immunization period.

Following challenge, foals appeared alert and in good condition until seven days after initial respiratory exposure. The control group became increasingly depressed and dyspneic from this time, and became anorexic. The CR- foals appeared somewhat depressed from days 7-9, but the CR+ group remained alert.

Mean temperatures, pulse and respiratory rates of foals in the three groups are shown in Table I. These parameters were significantly elevated in the nonimmunized foals compared to immunized animals from day 7 after initial infection. For humane reasons, all nonimmunized foals were euthanized on day 10, as were, for control reasons, one foal from each of the two immunized groups. The remaining foals were euthanized 14 days after first infection.

Significant pathological changes were observed only in the lungs. Nonvaccinated animals had heavy, edematous, congested lungs with extensive consolidation. On cut section, the consolidated parenchyma comprised multiple coalescing tan-colored, fleshy foci with no suppuration. In less severely affected areas, these foci were centrilobular in distribution. The lungs of one CR+ foal, sacrificed at the same time as the controls, showed areas of patchy consolidation, which were considerably less than in control animals. The CR- foal sacrificed at the same time as the controls had lungs which were moderately congested and slightly firmer on palpation than normal lungs.

The lungs of the two foals in each of the CR+ and the CR- groups killed 14 days after first aerosol challenge were congested but were otherwise normal.

TABLE I. Temperature, Pulse and Respiratory Rates in Immunized and Nonimmunized Foals Following Aerosol Challenge with *Rhodococcus equi*

Parameter; Foal Group[a]	Days Following Challenge						
	1	3	5	7	9	11	13
Temperature (C)							
CR+	38.6 ±0.7	39.3 ±0.1	38.5 ±0.2	37.9 ±0.4	38.1 ±0.2	38.0 ±0.1	38.0 ±0.2
Control	38.5 ±0.8	38.6 ±0.6	38.7 ±0.1	39.7 ±0.8[b]	40.5 ±0.3[b]	-	-
CR-	38.7 ±0.8	39.1 ±0.6	39.1 ±0.6	38.0 ±0.2	38.2 ±0.2	38.1 ±0.1	38.3
Respiratory rate (respirations/min)							
CR+	ND[c]	ND	ND	31 ±5	27 ±8	23 ±1	18 ±2
Control	ND	ND	ND	48 ±6[b]	67 ±30[b]	-	-
CR-	ND	ND	ND	37 ±6	25 ±2	28 ±2	20
Pulse rate (beats/min)							
CR+	ND	ND	ND	71 ±10	61 ±7	64 ±4	76 ±4
Control	ND	ND	ND	87 ±22[b]	99 ±23[b]	-	-
CR-	ND	ND	ND	65 ±10	61 ±8	75 ±5	72

[a]CR+, immunized with CR+ *R. equi* strain; Control, nonimmunized control; CR-, immunized with CR- *R. equi* strain. Three foals per group day 0-10, two thereafter. Control foals euthanized day 10
[b]Significantly different from other groups ($p<0.05$ Student's t-test)
[c]Not done

Histologically, the lungs of the control unvaccinated foals showed severe pyogranulomatous pneumonia, characterized by extensive infiltration of alveolar spaces by neutrophils, macrophages, and some multinucleate giant cells. Special stains demonstrated large numbers of gram-positive coccobacilli within macrophages and giant cells. While macrophage and neutrophil degeneration was evident, the lesions were not yet suppurative and interalveolar septa were largely intact.

The lungs of the vaccinates (CR+ group) were congested and slightly atelectatic. Interalveolar septa were generally hypercellular giving the appearance of a very mild interstitial penumonia. Peribronchial and perivascular lymphoid accumulations were prominent in some sections. The lungs of two of the CR- vaccinate group resembled those of the CR+ group. The lungs of the third CR- vaccinate had some patchy lesions resembling those described for the unvaccinated controls but with very prominent peribronchial, perivascular and interlobular lymphocytic accumulations.

The mean number of *R. equi* isolated from four lung sites in the three control foals killed on day 10 varied between 3.7 and 5.7 × 10^9 per gram. In contrast, the mean number of *R. equi* per gram of lung was 6.0 × 10^5 for the CR+ foal and 2.8 × 10^6 for the CR- foal killed on the same day. By day 14 the number of bacteria recovered from the vaccinated foals was negligible. The mean number of *R. equi* isolated from the two CR+ foals was 1.6 and 2.9 × 10^2 per gram and from the two CR- foals was 1.6 × 10^2 and 40 per gram respectively.

DISCUSSION

Oral immunization of foals with a low passaged lung isolate, and a high-passaged variant, was shown to be highly effective in protecting foals against experimental aerosol challenge with large numbers of *R. equi*. Earlier, field studies of parenteral vaccination of foals against *R. equi* by Magnusson (10), using killed bacteria, did not demonstrate a protective effect. Prescott and others (11) failed to protect foals with parenteral administration of an alum-adjuvanted bacterin against a subsequent intratracheal challenge.

Prescott and others (6) found that oral administration of live *R. equi* to foals resulted in a lymphocyte blastogenic response to *R. equi* antigens. A single oral administration did not result in gross intestinal pathological changes or clinical signs typical of *R. equi* infection although minor histological intestinal lesions following uptake of bacteria in the Peyer's patches were noted (12). They commented that ingestion of *R. equi* probably resulted in natural vaccination. This suggestion was supported by the work of Takai and others (5) which showed that specific IgG levels rose after oral administration of *R. equi*, but that detectable levels of specific IgA and IgM only developed after intracheal inoculation of this bacterium. IgG is the predominant immunoglobulin isotype in the lower respiratory tract (13). The mechanism of the protective immunity induced by oral immunization, whether it is humoral or cell-mediated, or both, remains to be elucidated. No attempt was made to detect naturally occurring maternally derived antibody in the foals, although at the age at which these animals were challenged such antibody might be expected to be at a minimum (4).

Pathological changes in the lungs of control foals were typical of the early lesions of experimentally induced *R. equi* pneumonia (2,9). The size of the aerosol challenge resulted in changes of subacute bronchopneumonia not typical of most cases of natural disease, but resembling those described after experimental aerosol infection of foals by Martens and others (9). A single aerosol infection might have been a more realistic challenge, but protection against the challenge dose used showed the high degree of immunity produced. This also contrasts with the lack of protection offered by parenteral vaccination with washed, killed bacteria in the face of a single intratracheal exposure (11).

The rapid slight (and not statistically significant) rise and fall in temperature of the two vaccinated groups in the first three days after challenge contrasted with the delayed rise in the control group, and was possibly the expression of immunological and inflammatory reactions in the first days after challenge. Clearance of *R. equi* from the lungs of the immunized foals occurred relatively rapidly. Ten days after initial challenge there was a 1000-10,000-fold difference in bacterial counts between controls and vaccinates. By day 14 vaccinates had few residual *R. equi* in the lungs.

No clear difference in protection was observed in the CR+ and the CR- strain, although the CR- strain may have been marginally less immunogenic, as one of the three CR- vaccinates developed significant lesions on challenge. We have observed (unpublished observations) that some CR- strains are less mouse virulent than their CR+ parents. Congo red staining in clinical isolates of *R. equi* correlates closely with the presence of two prominent protein bands on polyacrylamide gel electrophoresis (unpublished observations), but these proteins appear not to be important in protective immunity in foals. We did not, however, examine reversion from CR- to CR+ phenotype in the bacteria administered.

The immunization procedure appeared safe for the foals. Nevertheless the procedure described should not be used on farms with endemic *R. equi* pneumonia problems. Takai and others (14) have described the multiplication of *R. equi* which occurs in the intestine of the foal, and which peaks at about eight weeks of age. The administration of virulent *R. equi* by stomach tube to foals would rapidly result in dissemination of large numbers of these organisms into the environment. It is of interest that foals were reared on pastures used for horses for a number of years and very likely contaminated with *R. equi*. Such natural infection may not be adequate to protectively immunize foals in the critical period at which they appear susceptible to *R. equi* pneumonia. It will be necessary for artificial immunization to produce immunogenic strains which do not survive in the environment before oral vaccination can be recommended as an approach to control of the disease. Further studies should define the protective antigens and explore parenteral immunization for protection against disease. Nevertheless, the observation that oral immunization protects foals against *R. equi* pneumonia offers an encouraging approach to the control of this disease.

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Temperature-Sensitive Mutants of Type I *Streptococcus pneumoniae*: Preparation, Characterization, and Evidence for Attenuation and Immunogenicity

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Thirteen temperature-sensitive (*ts*) mutants of type I *Streptococcus pneumoniae* were selected after exposure of virulent wild-type (*ts*$^+$) organisms to nitrosoguanidine. Each mutant resembled the *ts*$^+$ parent in properties of α-hemolysis, bile solubility, optochin sensitivity, antibiotic sensitivity, and serotype. Unlike the *ts*$^+$ parent, however, each *ts* mutant was restricted in its capacity to form colonies on blood agar at 38 C. With the exception of two mutants, there was a correlation between the degree of temperature-sensitivity of a mutant and its genetic stability. When inoculated intraperitoneally into mice, 11 of 13 mutants were attenuated and induced homologous resistance. Three mutants (*ts* 1, *ts* 3, and *ts* 4) were also studied in hamsters and were found to be attenuated and immunogenic after intraperitoneal injection. Study of the behavior of mutants *ts* 1, *ts* 3, and *ts* 4 in the blood of hamsters suggested that attenuation may be related, in part, to decreased growth and survival of *ts* organisms at body temperature. Mutants *ts* 1 and *ts* 4 were completely attenuated for hamsters when administered intranasally and induced significant resistance to subsequent challenge with wild-type organisms by the same route. Local administration of *ts* mutants of type I *S. pneumoniae* to hamsters may provide a model for evaluating the potential of live vaccines in the prevention of disease due to bacterial respiratory tract pathogens.

With the recognition of the importance of local immune processes in resistance to viral and mycoplasmal respiratory diseases, emphasis has been placed upon the development of vaccines that stimulate local immunity when introduced into the respiratory passages. One promising approach is based upon the temperature gradient that exists within the respiratory tract. Temperatures range from 27 C in the outer nasal passages to 37 C in the lungs when the environmental temperature is 22 C [1, 2]. To take advantage of this temperature differential, mutants of respiratory tract pathogens have been selected that grow vigorously at 32 C but that do not replicate efficiently at 37 C–38 C in vitro [3–5]. Theoretically, temperature-sensitive (*ts*) mutants of this type should replicate efficiently at temperatures prevalent in the upper respiratory passages and should stimulate local immunity. However, because of genetic restriction on growth at the higher temperature of the lungs, the mutants should not be able to replicate to a titer sufficient to produce disease in the lower respiratory tract.

Because of the encouraging results obtained with *ts* mutants of viral and mycoplasmal respiratory tract pathogens, we wondered whether *ts* mutants of bacterial respiratory pathogens might also merit consideration as potential vaccine candidates. Such an alternate approach to immunoprophylaxis might be considered in the case of certain important bacterial diseases affecting

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the young, whose immunologic response to some parenterally administered purified polysaccharides is less than optimal [6–8]. These diseases include otitis media due to *Streptococcus pneumoniae* and *Haemophilus influenzae* and meningitis due to *S. pneumoniae, H. influenzae,* and group B *Neisseria meningitidis. S. pneumoniae,* the major bacterial respiratory tract pathogen of children and adults, was selected to test the feasibility of this approach. This paper summarizes the methods used to prepare and characterize *ts* mutants of type I *S. pneumoniae* and presents evidence of attentuation and immunogenicity of the mutants in experimental animals.

Materials and Methods

Type I S. pneumoniae. The wild-type or *ts*$^+$ strain of type I *S. pneumoniae* employed was isolated in 1935 from the sputum of a patient with lobar pneumonia, was passaged in mice, and was preserved by lyophilization. After reconstitution from the lyophilized state, the strain was passaged five times in mice and 10 times in hamsters [9]. Cloned organisms that were grown in blood agar and that were from the final passage in hamsters were suspended in brain-heart infusion broth with 10% glycerol and stored in 2-ml aliquots at −70 C for subsequent use.

Mutagenesis and selection. A logarithmic-phase culture of *ts*$^+$ *S. pneumoniae* was centrifuged, the pellet was suspended in phosphate-buffered saline (PBS, pH 7.4), and aliquots were dispensed into sterile centrifuge tubes. An appropriate volume of a freshly prepared solution of N-methyl-N′-nitro-N-nitrosoguanidine (Aldrich Chemical Co., Milwaukee, Wis.) was added to half the tubes to bring the concentration of mutagen to 50 μg/ml; an identical volume of PBS was added to the remaining tubes which served as controls. The tubes were then incubated at 37 C, and at 15- to 30-min intervals, bacteria were harvested, washed, and titrated on 5% sheep blood agar plates to obtain a bacterial killing curve. Mutants were selected from nitrosoguanidine-treated suspensions in which the bacterial killing was 10-fold greater than that in the untreated control. Fifty to 100 cfu from nitrosoguanidine-treated suspensions were spread on a series of blood agar plates that were incubated at 32 C for 24–48 hr and then used as templates for subsequent replica plating [10]. Two blood agar replicates of each template were prepared; one was incubated at 32 C (a permissive temperature) and the other at 38 C (a restrictive temperature). Colonies appearing on the 32 C plate without a counterpart on the 38 C plate were picked, streaked on blood agar, and tested again at the permissive and restrictive temperatures. Putative *ts* mutants were then cloned four times. Organisms grown in blood agar that were from the final cloning were frozen in brain-heart infusion broth with 10% glycerol.

Efficiency of colony formation. Serial 10-fold dilutions of suspensions of *ts*$^+$ and of *ts S. pneumoniae* were spread on blood agar plates that were incubated at 32 C, 34 C, 36 C, 37 C, 38 C, or 39 C. Colonies were counted after incubation for 48 hr.

In vitro genetic stability. The genetic stability of *ts* mutants of type I *S. pneumoniae* was examined on agar and in liquid media. The frequency of reversion on 5% sheep blood agar was estimated from the ratio of colony counts obtained after incubation for 48 hr at 32 C and 38 C (cfu_{38}/cfu_{32}). Clonal analysis indicated that colonies which developed at 38 C contained organisms that subsequently produced colonies with high efficiency at the restrictive temperature ($cfu_{38}/cfu_{32} = 1$); such organisms were therefore considered to be revertants. Reversion frequencies in trypticase soy and brain-heart infusion broths were estimated by tube dilution tests. Serial decimal dilutions of *ts* organisms were prepared in broth and incubated for 24 hr at 38 C. The reversion frequency was the smallest inoculum of *ts* organisms that induced development of turbidity and/or growth of pneumococci capable of colony formation on blood agar at 38 C.

Studies in mice. Groups of eight to 10 Swiss mice were inoculated ip with 0.2-ml volumes of decimal dilutions of pneumococcal suspension. Control mice were inoculated with heat-killed organisms (56 C for 2 hr) or broth alone. The LD_{50} was calculated on the basis of the cumulative mortality rate during the week after inoculation [11]. Mice inoculated one month previously with one of the *ts* mutants or broth were challenged ip with 200 LD_{50} of virulent *ts*$^+$ organisms,

and deaths were recorded for seven days afterwards.

Studies in hamsters. Male golden Syrian hamsters (80 g) were obtained from Charles River-Lakeview, Newfield, N.J. On day 0, hamsters were inoculated either ip or intranasally with 10^7 cfu of *ts*$^+$ or *ts S. pneumoniae* suspended in 0.2 ml of brain-heart infusion broth. There were 54 animals in each experimental group. Control animals were inoculated with broth or heat-killed organisms. Morbidity and mortality in control and experimental groups were recorded daily for one week after inoculation. On days 0, 1, 2, 3, 4, 5, and 7 after inoculation, three animals in each experimental group were exsanguinated. Sheep blood agar plates were immediately inoculated with decimal dilutions of unclotted hamster blood and incubated at 32 C for 48 hr; colony counts were used to quantitate pneumococcal bacteremia. Heart, lung, and peritoneal tissues were removed and fixed and stained for histological analysis. Antibodies to *S. pneumoniae* in serum were measured by a sensitive radioimmunoassay system [12].

Ten weeks after primary inoculation, hamsters were challenged intranasally with 10^7 cfu of *ts*$^+$ *S. pneumoniae* to determine whether homologous resistance had developed. Morbidity and mortality among experimental and control groups were monitored for two weeks after challenge.

Results

General properties of ts mutants of S. pneumoniae. A total of 13 *ts* mutants of type I *S. pneumoniae* were selected in two separate mutagenesis experiments. The *ts* mutants were phenotypically similar to the wild-type parent in properties of α-hemolysis, bile solubility, and optochin sensitivity [9]. The mean bactericidal levels of penicillin and erythromycin for *ts* organisms were essentially identical to those for *ts*$^+$ organisms [9]. In addition, each of the mutants retained type I immunological properties, as determined by agglutination and Quellung tests [9]. Capsular polysaccharides derived from the *ts* mutants were immunochemically similar to each other and to those of the *ts*$^+$ parent, as indicated by immunodiffusion analysis and by competitive antigen binding [9].

Efficiency of colony formation and genetic stability of ts mutants of S. pneumoniae. The efficiency of colony formation by three representative mutants on blood agar at several different incubation temperatures is compared with that of the *ts*$^+$ parent in figure 1. The shutoff temperature was defined as the lowest temperature at which a ≧1,000-fold reduction in colony-forming capacity occurred. Whereas colony formation by *ts*$^+$ organisms was essentially unaffected by a temperature of as high as 39 C, the three *ts* mutants exhibited a spectrum of temperature sensitivity; *ts* 1 shut off at 38 C, *ts* 4 at 37 C, and *ts* 3 at 36 C.

The relation between the temperature sensitivity of a mutant (i.e., shutoff temperature) and in vitro genetic stability is shown in figure 2. It should be noted that detection of revertants was dependent in part upon the medium employed. Mutants *ts* 2, *ts* 9, and *ts* 7, which were the least temperature-sensitive (shutoff temperature,

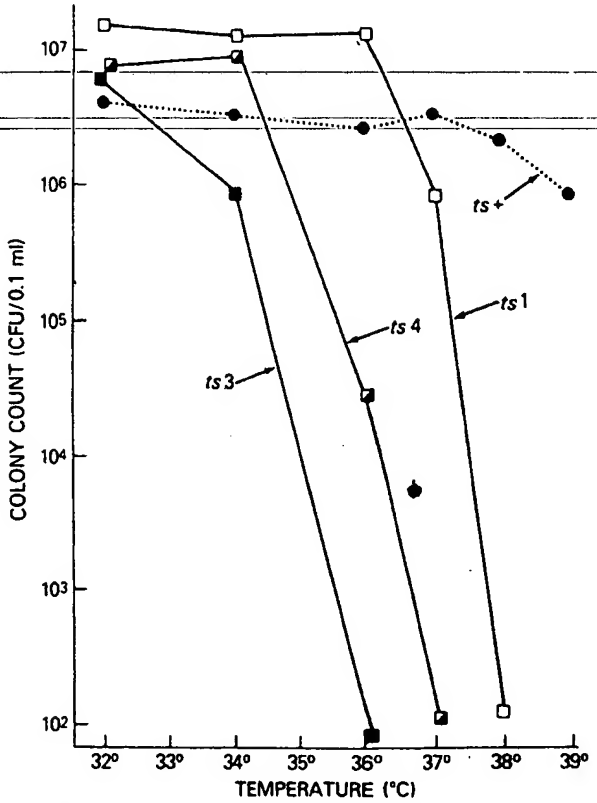

Figure 1. Efficiency of colony formation by temperature-sensitive (*ts*) and wild-type (*ts*$^+$) type I *Streptococcus pneumoniae* on 5% sheep blood agar at different temperatures.

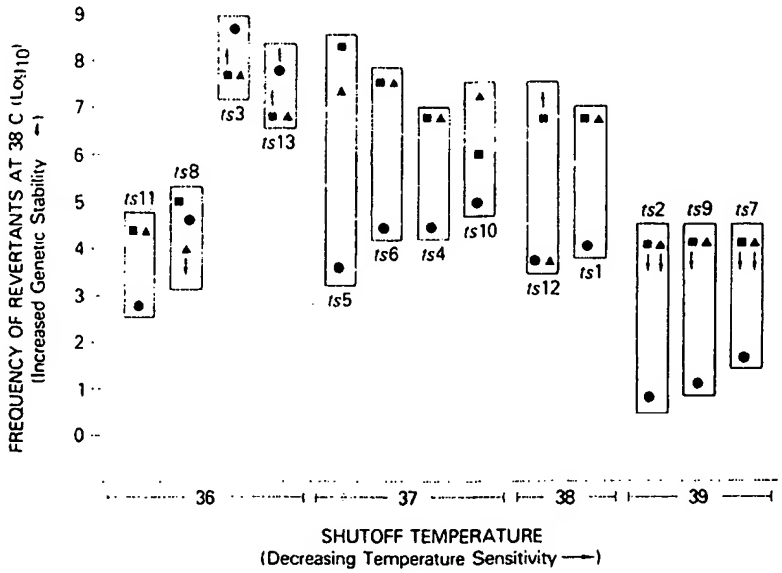

Figure 2. Relation of shutoff temperature of temperature-sensitive (*ts*) mutants of type I *Streptococcus pneumoniae* to in vitro genetic stability. Detection of revertants was in part dependent upon the medium employed: (●) = 5% sheep blood agar; (■) = trypticase soy broth; (▲) = brain-heart infusion broth.

39 C), were also the least stable. Mutants *ts* 1, *ts* 4, *ts* 5, *ts* 6, *ts* 10, and *ts* 12, which were of intermediate temperature sensitivity (shutoff temperature, 37 C–38 C), were of intermediate genetic stability. Mutants *ts* 3 and *ts* 13, which were among the most temperature-sensitive of the mutants (shutoff temperature, 36 C), were also the most stable genetically. Mutants *ts* 11 and *ts* 8, which were also among the most temperature-sensitive of the mutants, had intermediate genetic stability and thus were apparent exceptions to the general correlation between temperature sensitivity and genetic stability.

Virulence and immunogenicity of ts mutants of S. pneumoniae in mice. The virulence of *ts*$^+$ and *ts* type I *S. pneumoniae* administered ip to mice was assessed as shown in figure 3. The *ts*$^+$ parent, with a shutoff temperature of >39 C, was highly lethal with an LD_{50} of $10^{0.2}$ cfu. Significantly, 11 of the 13 *ts* mutants were markedly attenuated; these attenuated mutants had an LD_{50} that ranged from 10^5 to 10^7 cfu. There was no clear-cut relation between shutoff temperature and virulence.

Mice inoculated one month previously with 10^4—10^7 cfu of one of the 11 attenuated *ts* mutants were challenged ip with a lethal dose of *ts*$^+$ organisms to determine whether resistance had been induced. The number of mice that were challenged in each group ranged from 11 to 66. As shown in figure 4, 24%–100% of mice previously inoculated with a *ts* mutant survived a

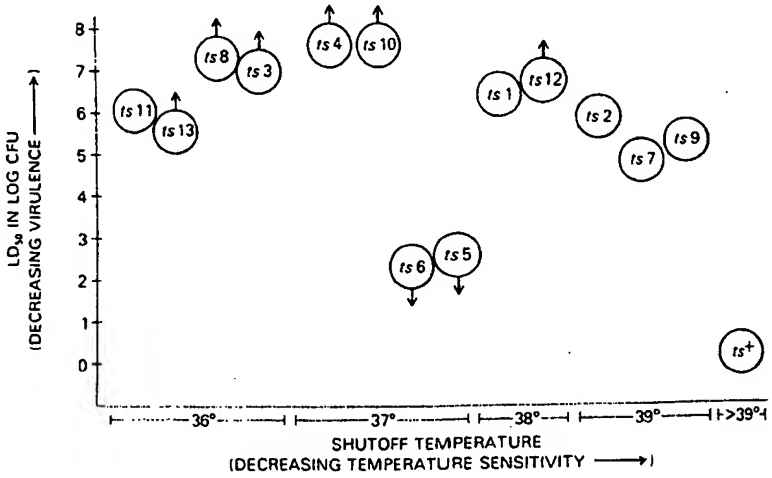

Figure 3. Virulence of temperature-sensitive (*ts*) mutants of type I *Streptococcus pneumoniae* administered ip to mice. (↑) = no mortality at highest dose tested; (↓) = no survival at lowest dose tested.

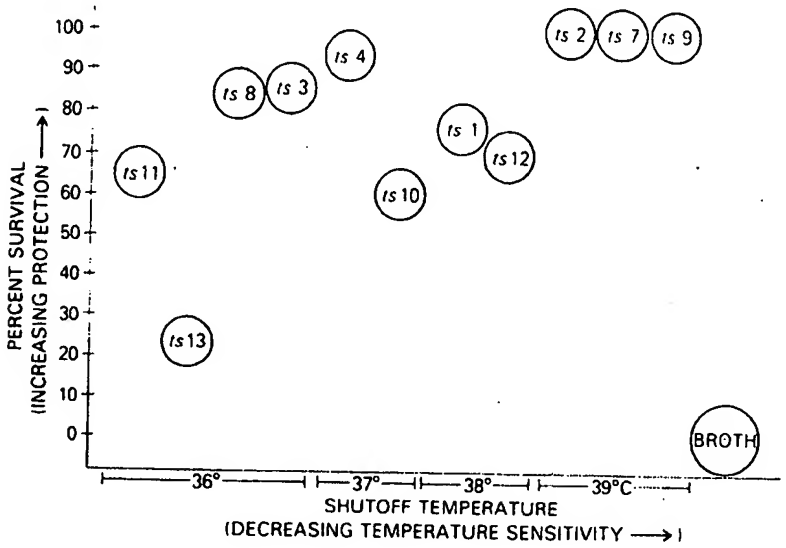

Figure 4. Resistance of mice inoculated ip four weeks previously with temperature-sensitive (*ts*) mutants of type I *Streptococcus pneumoniae* to lethal ip challenge with 200 LD_{50} of the wild-type (ts^+) organisms. All groups given *ts* mutants were significantly protected ($P < 0.001$, χ^2 analysis) as compared with broth-inoculated controls.

challenge with 200 LD_{50} of ts^+ organisms, whereas all of the control animals inoculated with broth died ($P < 0.001$, χ^2 analysis). Although there was no consistent relation between the shutoff temperature of a mutant and the protective effect induced in the mouse, mice that survived infection with mutants with a shutoff temperature of 39 C were uniformly resistant, whereas mutants with a lower shutoff temperature were more variable in their protective effect.

The relation between the residual virulence and immunogenicity associated with different doses of three selected *ts* mutants is shown in figure 5. The ts^+ parent was highly lethal, with 1

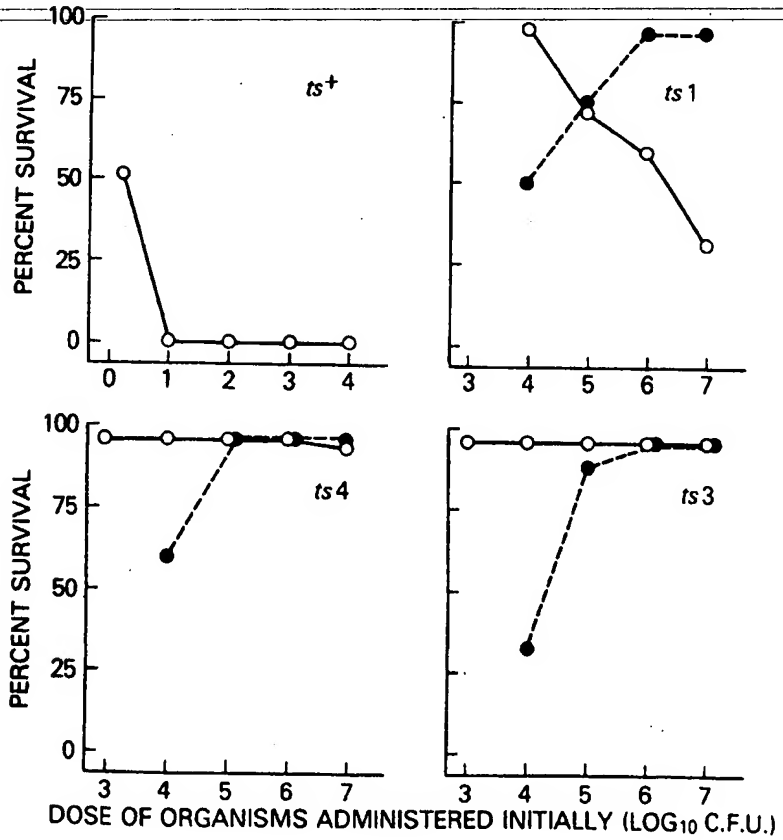

Figure 5. Response of mice to ip inoculation with temperature-sensitive (*ts*) or wild-type (ts^+) type I *Streptococcus pneumoniae* and to subsequent lethal ip challenge with ts^+ organisms. (○——○) = survival after primary inoculation; (●- - - -●) = survival after challenge with 200 LD_{50} of ts^+ organisms.

cfu producing a mortality rate of 50%. Mutant *ts* 1 produced significant dose-dependent mortality in inocula ranging from 10^5 to 10^7 cfu, but this effect was less than that seen with the wild-type parent. Inoculation of as many as 10^7 cfu of *ts* 4 or *ts* 3 organisms failed to induce significant mortality. Surviving mice were challenged ip one month later with 200 LD_{50} of *ts*$^+$ organisms. The dose of *ts* 1 organisms that induced complete resistance also produced significant mortality. In contrast, the dose of *ts* 4 and *ts* 3 organisms that induced complete or almost complete resistance to challenge with *ts*$^+$ organisms did not kill mice. Thus, there appeared to be a significant "safety factor" inherent in these two mutants.

Virulence and immunogenicity of ts mutants of S. pneumoniae in hamsters. Evidence of attenuation and immunogenicity of mutants *ts* 1, *ts* 4, and *ts* 3 was sought with use of another animal species, the golden Syrian hamster. Before inoculation, the mean rectal temperature of 10 lightly anesthetized hamsters was found to be 37.4 C (range, 36.2 C–38.3 C). It appeared that the extent and duration of bacteremia after ip inoculation of 10^7 cfu of *ts*$^+$ or *ts* organisms into hamsters was related to the shutoff temperature of the organism administered (figure 6). Thus, *ts*$^+$ *S. pneumoniae*, which shut off at a temperature of ≧1.6 C above the mean rectal temperature of the hamster, grew readily in the blood, attained peak concentrations in about 48 hr, and persisted for five days. Mutant *ts* 1, which shut off at 0.6 C above the hamster rectal temperature, failed to show an increase in titer in the blood but persisted at a detectable level for four days. Mutants *ts* 4 and *ts* 3, which shut off at 0.4 C and 1.4 C, respectively, below the hamster rectal temperature, failed to show an increase in titer and were detected only rarely, and then in low titer, after inoculation. Clearance of circulating organisms, whether *ts*$^+$ or *ts*, was associated with the appearance of type-specific antibodies by the third to fourth day, as detected by radioimmunoassay.

The degree of illness and histological evidence of disease observed after ip inoculation of 10^7 cfu of *ts*$^+$ or *ts* organisms paralleled the degree of bacteremia observed (table 1). Wild-type organisms reached levels of 10^8 cfu/ml in blood at 48 hr, and 90% of inoculated animals died. The rate of morbidity was 100%; 79% of the animals

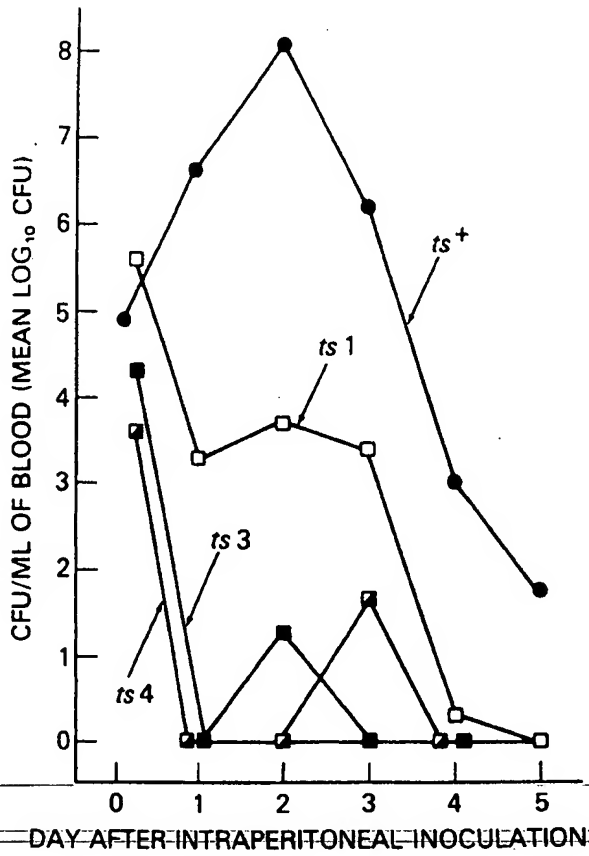

Figure 6. Bacteremia in hamsters after ip inoculation of 10^7 cfu of temperature-sensitive (*ts*) or wild-type (*ts*$^+$) type I *Streptococcus pneumoniae*.

developed pericarditis, and 88% had peritonitis. Mutant *ts* 1 produced a low level of bacteremia, less morbidity, and no mortality. Finally, mutants *ts* 4 and *ts* 3, which were rapidly cleared from the blood, failed to kill and produced very little morbidity.

Attenuation of the *ts* mutants was more striking when organisms were administered intranasally. Wild-type organisms, which reached levels of 10^7 cfu/ml in blood by 48 hr after inoculation, killed 60% of the inoculated animals, and those that did not die became ill; 38% of the hamsters developed pericarditis, and 33% had peritonitis. Unlike the *ts*$^+$ parent, each of the three *ts* mutants failed to produce bacteremia or disease and failed to stimulate the production of serum antibody by day 7.

Hamsters inoculated 10 weeks previously either ip or intranasally with 10^7 cfu of *ts S. pneumoniae* were challenged intranasally with 10^7 cfu of *ts*$^+$ organisms (table 2). Animals that were

Table 1. Response of hamsters to inoculation with 10^7 cfu of temperature-sensitive (*ts*) or wild-type (ts^+) type 1 *Streptococcus pneumoniae*.

Route of administration, organism	Bacteremia at 48 hr (mean $\log_{10}$ cfu/ml)	Mortality rate at one week (%)*	Morbidity rate at one week (%)*	Percentage of animals with: Pericarditis during first week†	Percentage of animals with: Peritonitis during first week†	Serum antibody response by day 7‡
Intraperitoneal						
ts^+	8.1	90	100	79	88	Yes
ts 1	3.8	0	64	14	33	Yes
ts 4	1.0	0	5	6	28	Yes
ts 3	1.3	0	0	0	11	Yes
Heat-killed §	None	0	0	5	0	Yes
Intranasal						
ts^+	7.4	60	100	38	33	Yes
ts 1	1.0	0	0	0	0	No
ts 4	1.0	0	0	0	0	No
ts 3	1.0	0	0	0	0	No
Heat-killed §	None	0	0	0	0	No

*Each group included 30–39 hamsters.

†Tissues were taken at daily intervals over seven days from a total of 13–21 animals in each group and examined histologically. The value reported is the cumulative percentage.

‡As measured by radioimmunoassay.

§Included ts^+, *ts* 1, *ts* 4, and *ts* 3 organisms.

previously inoculated ip with *ts* 1, *ts* 3, *ts* 4, or heat-killed organisms exhibited significant resistance to intranasal challenge with virulent organisms. Although hamsters failed to develop detectable serum antibody one week after intranasal administration of *ts* 1 or *ts* 4 organisms, such animals were demonstrably resistant to intranasal challenge with ts^+ organisms 10 weeks later. This finding suggests that the *ts* 1 and *ts* 4 mutants were able to infect when instilled locally into the respiratory tract and that this type of infection induced immunity. Animals that received living *ts* 3 organisms or heat-killed organisms (ts^+, *ts* 1, *ts* 4, or *ts* 3) intranasally were not resistant to subsequent intranasal challenge with ts^+ organisms.

Discussion

That the evaluation of *ts* bacterial mutants as a possible source of vaccines to prevent bacterial disease has been limited is surprising considering the extensive study of such mutants in molecular genetics. A temperature-sensitive filamentous mutant of *Salmonella enteritidis*, studied extensively in mice and rats, was found to be attenuated and to afford substantial protection against oral infection with *S. enteritidis* [13].

Effective polysaccharide vaccines for the prevention of pneumococcal pneumonia in adults have been developed [14]. The use of such vaccines in prevention of pneumococcal disease in infants and young children appears problematic, however, since serologic response in this age group to some parenterally administered polysaccharides is poor [6–8, 14]. Thus, live attenuated mutants of *S. pneumoniae* administered locally might provide an alternate approach to the immunoprophylaxis of pediatric pneumococcal disease, which occurs when immunologic responsiveness to polysaccharide vaccines is less than optimal.

In the present study *ts* mutants of type I *S. pneumoniae* were selected after treatment of virulent organisms with nitrosoguanidine. The mutants resembled the ts^+ parent in bacteriological and immunochemical properties and, more important, retained sensitivity to antibiotics. The *ts* mutants differed from the ts^+ parent, however, in that each of the mutants grew poorly or not at all at temperatures near that of the body core of humans, a characteristic that theoretically should restrict systemic growth. However, growth of the mutants occurred in vitro at temperatures comparable to those in the upper airways.

It is important that infection initiated by *ts*

Table 2. Response of hamsters inoculated 10 weeks previously with 10^7 cfu of temperature-sensitive (*ts*) type I *Streptococcus pneumoniae* to intranasal challenge with 10^7 cfu of wild-type (ts^+) organisms.

Route of primary inoculation, material inoculated (no. challenged)	Mortality rate at one week (%)	Morbidity rate during first week (%)
Intraperitoneal and intranasal, broth (40)	45	98
Intraperitoneal		
ts 1 (21)	0*	0*
ts 4 (23)	4*	4*
ts 3 (24)	0*	4*
Heat-killed (32)†	16*	41*
Intranasal		
ts 1 (21)	14*‡	14*‡
ts 4 (22)	9*‡	9*‡
ts 3 (21)	57	100
Heat-killed (28)†	60	96

*These rates were significantly less than the corresponding rates in broth-inoculated controls ($P < 0.05$, Fisher's exact test).

†Included ts^+, *ts* 1, *ts* 4, and *ts* 3 organisms.

‡These rates were significantly less than the corresponding rates for animals receiving heat-killed organisms intranasally ($P < 0.05$, Fisher's exact test).

mutants be sufficiently extensive to stimulate local and systemic immunity, yet be restricted enough to be clinically inapparent. Most of the *ts* mutants of type I *S. pneumoniae* examined in the mouse and the hamster appeared to meet these criteria. Of particular interest was the observation that mutants *ts* 1 and *ts* 4 were attenuated and protective when inoculated locally into the respiratory tract of hamsters. These encouraging results suggest that local administration of *ts* mutants of type I *S. pneumoniae* to hamsters may provide a model for evaluating the potential of live vaccine for the prevention of disease due to bacterial respiratory tract pathogens.

The question of the importance of the *ts* lesion or lesions in attenuation remains unanswered at this time; however, the relation of temperature sensitivity to the extent of bacteremia in a limited test of three mutants suggests that this property may be of significance in attenuation. It is very likely that attenuation is a composite property that is contributed to by mutagen-induced non-*ts* as well as *ts* mutations.

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SHORT COMMUNICATIONS

Immunization of Mice Against *Streptococcus suis* Serotype 2 Infections using a Live Avirulent Strain

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ABSTRACT

In this study, the IgG response of mice injected with two virulent strains and one avirulent *Streptococcus suis* capsular type 2 strain was compared by Western blotting. The serum from mice immunized against the avirulent strain could recognize most proteins of the various strains tested and similar results were obtained with serum from mice injected with virulent strains. The live avirulent strain was injected twice (days 0 and 10) to groups of five mice, and four virulent strains from different geographical origins were used to challenge the animals. All mice, except one in one group, survived the challenge. These results suggest that a live avirulent strain could be used for immunization of swine, the natural host.

RÉSUMÉ

Dans cette étude, la technique d'immunobuvardage a été utilisée pour comparer la réponse en IgG de souris auxquelles on a administré une souche avirulente de *Streptococcus suis* serotype 2 à celle de souris auxquelles on a administré deux souches virulentes du même sérotype. Le sérum des souris immunisées avec la souche avirulente a reconnu la plupart des protéines qui étaient reconnues par le sérum des souris immunisées avec les souches virulentes. La souche avirulente a été injectée aux jours 0 et 10 à des groupes de cinq souris. Les souris furent ensuite infectées en utilisant, pour les différents groupes, une des quatres souches virulentes provenant de différentes régions géographiques. Toutes les souris, sauf une dans un groupe, furent protégées contre l'infection. Ces résultats suggèrent que l'utilisation de cette souche avirulente devrait être considérée pour d'éventuels essais de protection chez l'hôte naturel, le porc, en utilisant la bactérie vivante.

Streptococcus suis capsular type 2 is an important swine pathogen, causing mainly meningitis, septicemia and arthritis (1). Attempts to control diseases with antibiotics and/or vaccination have often been disappointing, even if autogenous inactivated whole cell vaccines have shown promise (1). Successful passive immunization of mice using antisera directed against different *S. suis* proteins have been reported (2,3) but active immunization against *S. suis* cellular proteins of a given strain failed to protect mice against heterologous strains (4). Live *S. suis* strains have previously been used to protect pigs against the disease but several injections were necessary for good protection (5).

In a previous study, it was noted that the electrophoretic protein profile of an avirulent strain was similar to those of virulent isolates (6). The failure of some avirulent strains to cause disease may be related, at least in part, to their inability to increase capsule production *in vivo* (6). One aim of this study was to compare the IgG response of mice immunized with an avirulent strain and virulent strains of *S. suis*. Another objective was to evaluate the protective potential of this avirulent isolate using a murine experimental model of infection (7). This model has been used in pathological studies (8) and in attempts to predict virulence for the natural host (9).

Five *S. suis* capsular type 2 strains were used; the reference strain (735), isolated in Denmark, was provided by Dr. J. Henrichsen, Statens Seruminstitut, Copenhagen. One isolate from the United States, AAH4, was provided by Dr. Brad Fenwick, Kansas State University. One Mexican isolate, J590, was provided by Dr. Jose Luis Monter Flores, University of Toluca. Two isolates, 1591 and 1330, were from our collection. Strain 1330 was avirulent whereas the other four strains were virulent for mice and pigs (7).

We evaluated, by Western blotting, the ability of IgG produced against various strains to recognize proteins from homologous and heterologous strains. Sera of mice injected with 10^8 cells (formalin-killed) of strains 1330, 735 and 1591 were used. For Western blots, cells were cultured overnight in Todd-Hewitt broth at 37°C, harvested by centrifugation, washed and resuspended in 3 mL of K_2HPO_4 (0.1M, pH 7.0). Cells were then processed three times in a French press cell, treated with lysozyme (5 mg/mL), and the supernatants, containing cytoplasmic and membrane proteins, were recovered after centrifugation (12,000 × *g*, 20 min). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (7.5% acrylamide) was then performed (10) in order to separate cellular proteins. Following SDS-PAGE, material was transferred from the slab gel to the nitrocellulose

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FIGURE 1

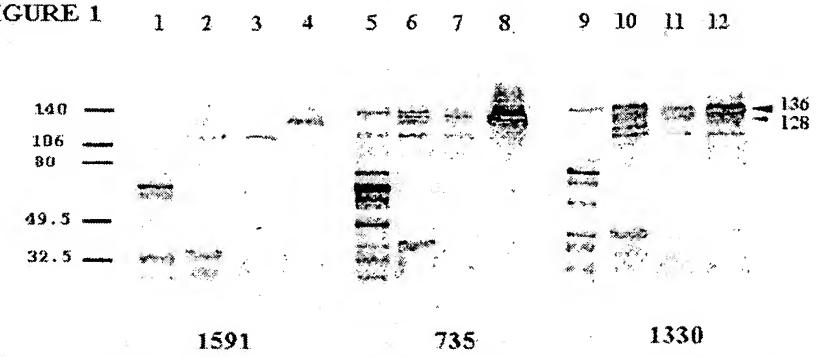

Fig. 1. Western blots of cellular proteins of *Streptococcus suis* capsular type 2 strains (7.5% acrylamide-SDS-PAGE). Protein profiles were revealed using mice antisera against strain 1330 (lanes 2, 6,10), strain 1591 (lanes 3, 7, 11) and strain 735 (4, 8, 12). Molecular weight markers in kDa. Below, strain identification numbers. Lanes 1, 5 and 9 were Coomassie blue stained proteins on acrylamide gels before transfer to nitrocellulose.

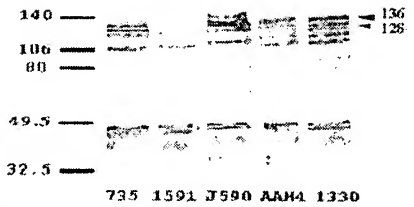

Fig. 2. Comparison, by Western blots, of cellular proteins of various *Streptococcus suis* capsular type 2 strains (7.5% acrylamide-SDS-PAGE) using mice antisera against strain 1330. Molecular weight markers in kDa. Below, strain identification numbers.

membrane by the methanol-Tris-glycine system (11) and the protein profiles were revealed using the various antisera and a peroxidase-labeled goat antiserum raised against murine IgG (Sigma Chemicals, St.Louis, Missouri) and 4-chloro-1-naphthol in cold methanol mixed with H_2O_2.

For immunization assays, four groups of five mice were injected intraperitoneally with 10^7 live cells of strain 1330 on days 0 and 14. Four other control groups were injected with PBS only. All groups of mice were challenged on day 21 by intraperitoneal injection of 10^8 cells of one of the four virulent strains. Western blots were repeated three times, the experiments with animals were repeated twice and the guidelines of the Guide to the Care and Use of Experimental Animals from the Canadian Council on Animal Care were followed.

The antisera raised against the various strains recognized most proteins of these strains and from two other virulent strains. However, some differences were detected in each strain using the different antisera. Mouse antisera against the avirulent strain recognized most proteins also recognized by antisera against the virulent strains (Fig. 1). This indicated that the mouse IgG response to the avirulent strain could recognize many proteins of virulent strains. In particular, the antiserum obtained by injecting mice with the avirulent strain recognized a protein of about 136 kDa in all virulent strains except one; this protein was not dectected in strain 1591 as previously noted (4,9), but was present in strain 1330 (Fig. 2). This 136 kDa protein was shown to be the most immunogenic *S. suis* cellular protein (4), and was recognized by a monoclonal antibody raised against the muraminidase released protein (MRP) (9,12).

Since the avirulent strain led to the production of IgG recognizing many proteins of virulent isolates and since antibodies directed against some proteinaceous epitopes had been shown to protect against the disease (4), it was suggested that antibodies directed against the avirulent strain proteins could induce protection against the virulent strains. Indeed, all mice from three groups were protected against mortality while four of five mice were protected in the fourth group (J590) (Table I). All mice, except one, died in the control groups.

Considerable genetic diversity has been found among *S. suis* isolates (13), but the avirulent strain succeeded in inducing a protection in mice against virulent strains from various geographical origins. Since a 110 kDa extracellular factor, previously reported as a virulence marker (12), was not detected in this strain it could indicate that this factor is not essential for protection.

In a previous study, the 136 kDa cellular protein was recognized by antibodies produced against a 128 kDa cellular protein, present in all strains (Fig. 1)(9). Since the sera of mice immunized with strain 1591, that does not possess the 136 kDa protein, recognized a 136 kDa in all other tested strains, it could also indicate that this strain possesses a protein, probably the 128 kDa protein, with epitopes shared by the 136 kDa protein.

Other structures, not considered in this study, such as capsular polysaccharides, may be involved in the immunity against *S. suis* but *S. suis* polysaccharides are poorly immunogenic (14). The IgG response of mice and pigs to *S. suis* capsular type 2 cellular proteins was shown to be similar (4). Thus, this study suggests that the avirulent strain 1330 would be a good candidate for vaccination of swine with live bacteria.

TABLE I. Active immunization of mice against virulent strains of *Streptococcus suis* serotype 2 using a live avirulent strain

Mice challenged[a] with *S. suis* strain	No. of sick mice[b]/ No. of injected mice (Control groups[c])	No. of dead mice/ No. of injected mice (Control groups)
735	0/5 (5/5)	0/5 (4/5)
J 590	1/5 (5/5)	1/5 (5/5)
1591	1/5 (5/5)	0/5 (5/5)
AAH4	0/5 (5/5)	0/5 (5/5)
1330	(0/5)	(0/5)

[a] Mice were injected intraperitoneally with 10^8 CFU of each strain one week following the second injection with strain 1330

[b] Mean numbers of mice which showed nervous signs and/or prostration during the week following the experiment. Results are the mean of two separate experiments

[c] Control groups were injected twice with PBS before the challenge

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Immunization of Pigs Against *Streptococcus suis* Serotype 2 Infection Using a Live Avirulent Strain

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ABSTRACT

Sreptococcus suis capsular type 2 is still an important cause of economic losses in the swine industry. At the present time, vaccination of pigs against this infection is generally carried out with autogenous bacterins and results are equivocal. In this study, the protective effect of a live avirulent *S. suis* type 2 strain (#1330) which had induced a good protection in mice, was evaluated in swine. The experiment was performed in triplicate using 4 week-old piglets. A total of 15 piglets were vaccinated 3 times, 15 others were vaccinated 2 times, and 15 piglets were injected 3 times with sterile Todd-Hewitt broth. Using an indirect ELISA, an increase in the IgG response to *S. suis* antigens was noted in 27 of the 30 vaccinated piglets. On day 21 post-vaccination, all animals were challenged intravenously with a virulent *S. suis* type 2 strain (#999). In the 2 vaccinated groups, 26 animals were fully protected. Only 1 out of the 15 piglets vaccinated 3 times developed mild clinical signs. In the group vaccinated twice, 3 piglets showed clinical signs and 1 of them died after the challenge. In the control group, 7 animals died out of the 11 with clinical signs of infection. In conclusion, a protective immunity was observed in swine when using strain 1330. However, more studies are needed to assess the use of a live *S. suis* strain in a vaccine for pigs.

RÉSUMÉ

Les infections dues à *Sreptococcus suis* sérotype 2 sont toujours une cause importante de pertes économiques pour l'industrie porcine. Jusqu'à maintenant, la majorité des essais de vaccination ont été effectués avec des bactérines autogènes et les résultats sont très mitigés. Cette étude avait pour objectif d'évaluer la capacité d'une souche vivante et avirulente de *S. suis* type 2 (#1330) d'induire une protection chez le porc. Cette souche souche avait auparavant induit une protection chez la souris. Dans la présente étude, comportant trois expériences répétées avec des porcelets âgés de quatre semaines, un nombre total de 15 porcelets ont été vaccinés trois fois, 15 autres ont été vaccinés deux fois et 15 porcelets ont reçu trois injections du bouillon de culture stérile Todd-Hewitt. À l'aide d'un test ELISA indirect, une augmentation du titre d'anticorps contre les antigènes de *S. suis* a été notée chez 27 des 30 porcelets vaccinés. Au jour 21 post-vaccination, tous les animaux ont reçu, par voie intra-veineuse, une injection de défi avec une souche virulente de *S. suis* sérotype 2 (#999). Dans les deux groupes d'animaux vaccinés, 26 porcelets sur 30 ont été protégés complètement. Parmi ceux ayant reçu trois doses du vaccin, un seul porcelet a manifesté des signes cliniques. Dans le groupe d'animaux vaccinés deux fois, trois porcelets ont présenté des signes cliniques et l'un d'eux est mort après l'injection de défi. Dans le groupe des témoins, 11 animaux ont présenté des signes cliniques et sept d'entre eux sont morts. En conclusion, une immunité protectrice a été observée chez l'espèce porcine lors de l'utilisation de la souche 1330 comme vaccin. Toutefois, d'autres études sont nécessaires avant de permettre l'utilisation d'une souche vivante de *S. suis* comme vaccin.

INTRODUCTION

Streptococcus suis is an important pathogen of swine causing mainly septicemia, meningitis and endocarditis. Serotype 2 is the most common capsular type recovered from cases of meningitis in weaned pigs in the United Kingdom, North America and the Netherlands (1–3). It is also associated with various types of infections in different animal species, as well as in humans (4,5). Economic losses due to *S. suis* are important and conventional control measures, such as vaccination, have so far given unsatisfactory results (6). Holt et al (7) found that numerous repeated vaccinations with inactivated cells were needed to induce a good protection. Injections of purified capsular polysaccharides in pigs failed to induce adequate protection (8) and poorly encapsulated strains appeared to be as immunogenic as fully encapsulated ones (9). However, passive and active immunization using different cell wall proteins succeeded in protecting mice against the infection (10–12). A few *S. suis* capsular type 2 strains were recently shown to be avirulent in pigs as well as in the mouse model of infection (13). One of them, strain 1330, harbored a highly immunogenic 135 kDa protein which is also present in virulent isolates. It succeeded in inducing a complete protection in mice against the experimental infection with virulent strains (14). The purpose of this study was to monitor the protective effect of vaccination with *S. suis* avirulent strain 1330 and

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TABLE I. Evaluation of antibody response and protection induced by vaccination of piglets with the live avirulent *Streptococcus suis* serotype 2 strain 1330

Experiment	Vaccination protocol	Mean of titer increase[a]	No. of sick pigs[b]/ No. of challenged pigs	No. of dead pigs[c]/ No. of challenged pigs	No. of pigs with *S. suis* in tissues[d]/ No. of challenged pigs
1	3 doses	5.4	0/5	0/5	0/5
	2 doses	3.5	0/5	0/5	0/5
	control	0	4/5	4/5	4/5
2	3 doses	8.5	0/5	0/5	0/5
	2 doses	2.5	2/5	1/5	1/5
	control	0	4/5	2/5	3/5
3	3 doses	8.8	1/5	0/5	0/5
	2 doses	1.8	1/5	0/5	0/5
	control	0	3/5	2/5	2/5

[a] Value represents mean antibody titer increase of animals from 1 group
[b] Nervous signs, lameness, and decubitus for more than 12 h were considered
[c] Number of euthanized animals showing decubitus or nervous signs for more than 12 h
[d] Bacteriological analyses following post-mortem examination; presence of *S. suis* in at least 1 organ or in blood

to study the antibody response in the natural host.

MATERIALS AND METHODS

BACTERIAL STRAINS AND GROWTH CONDITIONS

Two *S. suis* capsular type 2 strains were used in this study. Strains 999 and 1330 were isolated from pig tissues in the laboratory of clinical bacteriology of the Faculty of Veterinary Medicine, University of Montreal. They were identified as *S. suis* capsular type 2 using a procedure already described (2). These strains had previously been tested with an experimental mouse model of infection and in pigs (12,15) and their virulence estimated as follows: 999 highly virulent and 1330 avirulent. For each strain, 3–4 colonies from a 24 h culture on blood agar plates (5% bovine blood) were inoculated in Todd-Hewitt broth (THB) (Difco Laboratories, Detroit, Michigan, USA) and grown overnight. From this broth, 500 µL were added to 50 mL of fresh medium and grown without agitation at 37°C with 5% CO_2 until the desired absorbance (540 nm), or number of bacteria, was reached.

IMMUNIZATION OF PIGS

Upon their arrival, all pigs were tested serologically for the presence of antibodies against *S. suis* serotype 2 antigens. Using an ELISA assay, piglets with low levels of antibodies were kept for the study. Fifteen 4 week-old crossbred piglets were alloted to each of 3 separate, but identically designed experiments (Table I). In each experiment, pigs were divided into 3 groups of 5 animals and immunizations were carried out via the intra-muscular route. In group 1, 5 piglets were injected 3 times with 10^9 colony forming unit (CFU) of strain 1330 (days 0, 7 and 14). In group 2, 5 other piglets were injected 2 times with the same bacterial concentration (days 0 and 14). In group 3, 5 control animals were injected 3 times with sterile THB (days 0, 7, 14). Between days 0 and 21, pigs were examined twice daily to detect clinical signs of infection. Seven days before the first immunization and 7 d after the last immunization, 2 mL of blood were collected in order to evaluate the antibody response to *S. suis* capsular type 2 cellular proteins by ELISA and to some specific *S. suis* proteins by Western blot. Guidelines from the Guide to the Care and Use of Experimental Animals from the Canadian Council on Animal Care were followed during the experiment.

EXPERIMENTAL INFECTION AND ASSESSMENT OF CLINICAL SIGNS

Pigs were challenged on day 21 by intravenous injection of 10^8 CFU of strain 999 as described by Quessy et al (13). Experimental infection was carried out for a period of 10 d and clinical signs were monitored twice daily for 1 h. Recorded clinical signs included lameness, persistent lateral or ventral decubitus, fever (> 40.5°C), as well as nervous signs such as incoordination, paddling, and opisthotonos. Pigs in decubitus or manifesting nervous signs for more than 12 h, were euthanized for ethical reasons. A necropsy was performed on these animals and tissues such as blood, lung, liver, spleen and brain were cultured for the presence of *S. suis* according to a procedure already described (2). Remaining pigs were euthanized after the 10 d period and submitted for necropsy, where special attention was paid to the immunization site in order to detect any lesions caused by intramuscular injection of the vaccinal strain.

SDS-PAGE

Twenty-five milliliters (mL) of an overnight culture of the strain 999 were centrifuged (12 500 x *g*) for 20 min and resuspended in 1 mL of K_2HPO_4 (0.1 M, pH 7.0), processed in a French Press (16) (SLM, Amico, Urbana, Illinois, USA) (Mini-cell, 20 000 PSI, 3 times), treated with lysozyme (5 mg mL^{-1}) for 4 h at 37°C and centrifuged again (12 500 x *g*) for 20 min at 4°C. Cellular proteins present in the supernatant were harvested, mixed with equal volume of solubilization buffer, boiled 4 min and processed in 10% polyacrylamide vertical slab gels (with 4.5% stacking gels) (17). Gels were stained with Coomassie blue or transferred to nitrocellulose.

WESTERN BLOT ANALYSES

Following SDS-PAGE, material was transferred to the nitrocellulose membrane by electroblotting in a transblot apparatus (Bio-Rad, San Francisco, C) with methanol-Tris-glycine buffer for 1 h at 100 volts (18). Non-reacting sites on nitrocellulose membrane were blocked for 1 h with casein 2% (w/v). The membrane was incubated for 1 h with 1:200 (v/v) dilutions of each pig serum before and after vaccination. After washing, the

membrane was incubated for 1 h with a peroxidase conjugated goat anti-pig IgG (Jackson Immuno Research,West Grove, Pennsylvania, USA). After washing, the presence of bound antigens was visualized by reacting the nitrocellulose membrane with 0.06% 4-*chloro*-1-naphtol (Sigma) in cold methanol mixed to 0.02% H_2O_2 in Tris-NaCl. Apparent molecular weights were calculated by comparison with standards of known molecular weight (Bio-Rad).

SEROLOGICAL RESPONSE OF PIGS FOLLOWING VACCINATION

Porcine sera were tested using an ELISA procedure. Flat-bottom polystyrene microtiter plates (NUNC Immunoplates, Copenhagen, Denmark) were coated at 4°C for 18 h with 0.4 μg of protein extract (strain 1330) in 100 mL of 10 mM phosphate buffer, pH 7.4 (PBS) per well. Then, 100 mL of PBS containing 0.3% (w/v) of casein and 0.005% (v/v) of Tween 20 (Sigma) were added and left 1 h at room temperature in order to block free sites; plates were then washed 3 times. Pig sera were diluted serially in PBS, added in 100 μL amounts to appropriate wells and incubated for 1 h at room temperature. Serum from an axenic pig was used as negative control. The positive control was an anti-strain 999 hyperimmune pig serum obtained from a pig after 6 consecutive immunizations with inactivated bacteria. Well contents were discarded and the plates washed. A volume of 100 μL of goat anti-pig IgG conjugated to horseradish peroxidase (Jackson Immuno Research) diluted 1:2000 in PBS was added to each well and left for 1 h at room temperature. The plates were washed and 100 μL of 0.4 mM 2,2'-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid) (Sigma) dissolved in 0.05 M citrate buffer (pH 4.0) with 0.5 M H_2O_2 were added to each well. The absorbance was measured after 30 min of incubation at 22–23°C. Optical density was corrected by substraction of background binding in control wells (coated with PBS). ELISA titers were estimated as the highest dilution that gave an increase in light absorbance at 414 nm (A_{414}) more than twice the mean of the corresponding blank values (without antibody but with conjugate and substrate). Increase in antibody titer was calculated using this formula: titer obtained on day 21 divided by titer obtained on day 0. The mean of titer increase was calculated using this formula: total titer increase for each animal in the same group divided by the total number of pigs in this group.

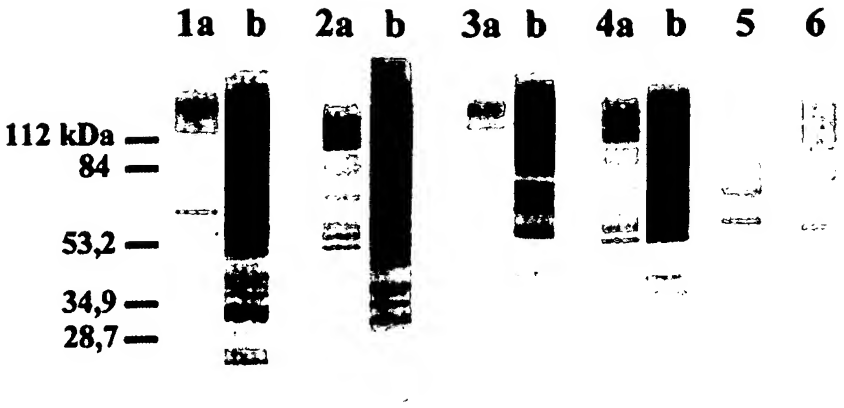

Figure 1. Comparison of the IgG response of pigs to *Streptococcus suis* proteins before (a) and after (b) immunization with the live avirulent strain 1330 by Western blot analysis. Piglets were immunized 3 times (lanes 1 and 2) and 2 times (lanes 3 and 4). Sera from control animals after they received 3 immunizations with sterile Todd-Hewitt broth (lanes 5 and 6). Results using antisera from animals selected from experiment 1 are shown; data were similar in experiments 2 and 3. Western blots were performed after transfer from a 12.5% polyacrylamide gel loaded with virulent strain 999 proteins.

RESULTS

IMMUNIZATION AND SEROLOGICAL RESPONSE OF PIGS

None of the pigs showed lameness, decubitus or nervous signs following immunization with the vaccinal strain of *S. suis*. Necropsy did not reveal any detectable lesions at the immunization site. In the first experiment, a 5.4 fold mean elevation in antibody titer against *S. suis* antigens was observed in pigs that received 3 doses of vaccine (Table I). Antibody titers between 40 000 and 80 000 were observed in these animals, while in animals vaccinated twice, antibody titers varied between 10 000 and 40 000, which corresponds to a 3.5 fold increase. In the control group, titers ranged from 5 000 to 10 000. A similar increase in the mean titer was observed in the 2 other experiments.

WESTERN BLOT ANALYSES

Western blot analyses carried out on sera from immunized pigs, before and after vaccination, showed an apparent increase in the IgG response against the virulent strain 999 cellular proteins (Figure 1 for experiment 1). An IgG response was observed against high molecular weight proteins and against proteins of approximately 40 and 70 kDa (Figure 1). Western blot analyses performed on sera in experiments 2 and 3 gave similar results (data not shown).

EXPERIMENTAL INFECTION

In the 3 experiments, when challenged by an intravenous injection of virulent strain 999, 1 out of the 15 pigs vaccinated 3 times and 3 out of the 15 pigs vaccinated twice showed clinical signs compatible with *S. suis* infection. In contrast, 11 out of the 15 control pigs manifested lameness, decubitus or nervous signs. Finally, 1 out of the 30 immunized pigs and 9 out of the 15 control pigs died (Table I). Lesions attributed to *S. suis* infection were observed and the microorganism was recovered from at least one organ or in blood from all animals having shown decubitus and/or nervous signs. Pathological lesions, and isolation of *S. suis* were recorded in only one of the other pigs (Table I).

DISCUSSION

Previous attempts to protect pigs against *S. suis* type 2 infection, either by injection of formalin killed bacteria (6,7,19) or purified capsular

material (8), have given equivocal results. However, Holt et al (9) using live cultures of *S. suis* induced a protective response in pigs after 8 consecutive immunizations. On the other hand, Quessy et al (14) succeeded in protecting mice against *S. suis* serotype 2 infection after only 2 injections with the live avirulent strain 1330. This strain, when incubated in intra-peritoneal chambers in rats, did not show any increase in the thickness of its capsular material (20). In contrast, when grown in similar conditions, an increase in the thickness of the capsular material was noted for virulent strains and was accompanied by a better resistance to killing by porcine polymorphonuclear leukocytes (12). In mice, it was previously demonstrated that immunization with the avirulent strain 1330 led to the production of IgG recognizing many proteins of different virulent strains (14).

A persistent high-level bacteremia usually precedes the onset of bacterial meningitis (21). Thus the ability of a bacteria to induce and maintain a bacteremia is a major determinant of pathogenicity (22). In this kind of infection, humoral immunity plays an important role. Moreover, the importance of humoral immunity in the pathogenesis of *S. suis* infection was confirmed by Holt et al who found that the protective response was serum-mediated and associated with both IgM and IgG (23). Presence of antibodies and particularly IgG at the bacterial surface could increase recognition and then stimulate uptake by phagocytes (24). However, the outcome of interactions between bacteria and phagocytes is important in determining the level of bacteremia and the incidence of meningitis (25). Since replication of virulent strains of *S. suis* within murine macrophages was shown by Williams (22), cellular immunity could also be determinant. Thus, in a live vaccine, organisms act as endogenous antigens and tend to trigger a response dominated by cytotoxic T-cells (26). In contrast, inactivated organisms, act as exogenous antigens and stimulate a response dominated by helper T-cells (26). If *S. suis* can survive inside phagocytes, both types of immunity would be required to eliminate all bacteria. Since activation is important in the control of organisms inside phagocytes, the control of *S. suis* infections would be improved with T-cell-mediated immune response, along with antibodies. Live bacteria are much more capable of activating phagocytes than inactivated organisms (26). The fact that a 1.8 fold increase in mean titer protected animals in the 3rd part of our assays would indicate that cellular immunity would have contributed, in a large proportion, to the protection observed.

In conclusion, protection against *S. suis* type 2 infection was observed in pigs after 2 or 3 vaccinations with the live avirulent strain 1330. This protection seems to be related to the presence of antibodies against some of *S. suis* cellular proteins and to cellular immunity. However, more studies are needed to assess the use of a live strain of *S. suis* in a vaccine for pigs.

ACKNOWLEDGMENTS

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Immune modification of the pathogenesis of *Streptococcus uberis* mastitis in the dairy cow

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Abstract: Two groups of 4 cows were vaccinated subcutaneously with live *Streptococcus uberis* strain 0140J or a surface extract derived from the same strain, at 14 days prior to the cessation of lactation (drying off) and at calving. Both groups also received an intramammary administration of the surface extract 7 days after drying off. A third group of unvaccinated animals acted as controls. Following intramammary challenge of two quarters per cow with the vaccine strain, all quarters on control cows and those vaccinated only with surface extract developed clinical mastitis. However, only 12.5% of challenged quarters on cows which were vaccinated with live bacteria developed clinical mastitis. In addition, the numbers of bacteria in the milk following challenge were 10^5 times higher from the control and extract vaccinated cows than those which received live vaccine. Serum levels of *S. uberis* specific IgG_2 were elevated in the animals vaccinated with the live organism when compared to that of either extract-vaccinates or controls, whilst *S. uberis* specific levels of IgG_1 and IgM were similar in all groups throughout the experiment. Specific antibody levels in milk were unaffected by vaccination. Despite increased levels of IgG_2, no increase in opsonic activity was detected in any serum or milk samples. Peripheral blood lymphocytes from animals vaccinated with live organisms showed a considerable increase in proliferative response to *S. uberis* antigen in vitro when compared with lymphocytes from control and extract-vaccinated animals. These results suggest that neutrophils and specific opsonising antibody may not form the major defence against infection with *S. uberis*.

Key words: Streptococcus uberis; Mastitis control; Vaccination; Lymphocyte stimulation

Introduction

Although control recommendations have effectively reduced mastitis incidence due to the contagious mammary pathogens such as *Staphylococcus aureus, Streptococcus agalactiae* and *Streptococcus dysgalactiae*, they have proved inadequate to reduce the rate of new infections caused by *Streptococcus uberis*. At least 20% of all cases of clinical mastitis are now due to this organism [1], and in many herds it is the major cause of the disease. A general failure to control mastitis caused by organisms of environmental origin has prompted the search for alternative control methods. Improvements in the understanding of bacterial physiology and virulence, together with studies of immunological processes and antigen presentation within the mammary gland, has resulted in renewed efforts to produce mastitis vaccines

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[2–4]. Nevertheless, attempts to produce a vaccine against mastitis-causing pathogens continue to be frustrated, not only by the number of different pathogens which can gain access to the mammary gland, but also by the difficulty in generating an appropriate and effective immune response. Systemic vaccination of sheep with live *Staphylococcus aureus* has been shown to generate high levels of IgG_2 and an enhanced neutrophil response, whereas sterile vaccines promoted the production of IgG_1 and were ineffective [5]. In cattle, the rapid and effective mobilisation of neutrophils has also been shown to be important in the defence against *Escherichia coli* mastitis [6,7] and to some extent against *Streptococcus agalactiae* infections [8]. However, the role of the neutrophil and opsonising antibody in the elimination of *S. uberis* from the mammary gland has not been established.

Previous studies showed that a primary infection with *S. uberis* can considerably reduce the rate of infection following a second challenge with the same strain [9]. Experiments also showed that some strains of *S. uberis* were significantly more resistant to phagocytosis by bovine neutrophils following growth in the presence of milk whey, casein or casein-derived amino acids [10], suggesting that bacteria growing in the lactating mammary gland may elaborate a pathogenic determinant in the form of an antiphagocytic factor.

In this report we describe the immunological response to vaccination with either live *S. uberis* grown in the presence of casein or an enzymically produced surface antigen preparation of similarly grown bacteria, together with its effect on the pathogenesis of experimentally induced intramammary infections with *S. uberis*.

Materials and Methods

Animals

Twelve Friesian cattle, from the Institute's dairy herd, and at the end of their first to third lactations, were used in the experiment. The animals selected had no history of clinical *S. uberis* mastitis. All animals were milked twice daily at 07.00 and 15.00 hours.

Bacteria

S. uberis strain 0140J (originally isolated from a case of bovine mastitis at the National Institute for Research in Dairying, Shinfield) was used throughout the experiment. The organism was stored in Todd-Hewitt broth (Oxoid) at −20°C in the presence of 25% (v/v) glycerol. For inoculation of defined medium or infection of cattle, bacteria were grown in Todd-Hewitt broth at 37°C for 18 h.

Vaccine preparations

(a) Bacterial surface extract. Chemically-defined medium (CDM), [10], containing glucose (1% w/v) and casein hydrolysate (BDH, 1% w/v) was inoculated to a density of approx. 1×10^6 colony forming units (cfu) ml^{-1} with a washed overnight culture of *S. uberis* 0140J and incubated at 37°C. Growth was monitored by measuring the optical density (550 nm) of aseptically removed samples. Bacteria (approx. 1×10^9 cfu ml^{-1}) were harvested during late exponential growth by centrifugation (12000 $\times g$, 30 min) at 4°C and washed twice in an equal volume of phosphate buffered saline (PBS). Washed bacteria were resuspended in 1.0% of the original culture volume of protoplasting buffer [11].

The bacterial cell walls and capsules were removed by incubation at 37°C for 60 min in the presence of mutanolysin (Sigma) and type X hyaluronidase (Sigma) at concentrations of 250 U ml^{-1} and 0.1 U ml^{-1} respectively. The resulting protoplasts were removed by centrifugation (7000 $\times g$, 20 min) at room temperature and the supernate (surface extract) carefully removed, filter sterilized (0.2 μm poresize) and stored in 1.0-ml aliquots at −20°C until required.

(b) Live bacteria. Bacteria were grown and harvested as described for the surface extract. After washing they were resuspended in 10% of the original culture volume of PBS and stored in 1.0-ml aliquots at −20°C until required. No loss in viability was detected following storage.

For subcutaneous administration, 1.0 ml of either surface extract or live bacteria was mixed with 1.0 ml of Freunds incomplete adjuvant (Difco) and 2.0 ml of Tween 20 (1% w/v). Immediately before use the vaccine was emulsified by

repeated expulsion through a 20 g hypodermic needle.

Vaccination regimes

The 12 cows were divided randomly into 3 groups of 4 animals. The control group had no vaccinations prior to infection. The second group (Live) was vaccinated sub-cutaneously, 14 days before the cessation of milking (drying off) and again within 24 h after calving, with 1×10^{10} live *S. uberis* strain 0140J. The third group (Extract) was vaccinated at the corresponding time and by the same route with the bacterial surface extract prepared as above. Seven days into the dry period both vaccinated groups received an intramammary infusion of 5 ml of the surface extract (20% v/v in sterile PBS) per quarter. Jugular blood samples were collected into glass universals for the preparation of serum and into sterile plastic universals containing 10 IU heparin (Evans) ml^{-1} blood where indicated. Milk samples were collected asceptically from each quarter where indicated.

Measurement of specific antibody by ELISA

Levels of specific antibody in serum were determined by ELISA. Bacteria were grown in supplemented CDM and the bacteria together with the medium stored in aliquots at −70°C prior to dilution in carbonate buffer (0.05 M, pH 9.6) to give 1×10^7 cfu ml^{-1}. Flat-bottomed microtitre trays (ICN, Flow Laboratories) were coated with 50 μl of the bacterial suspension overnight at 4°C. The plates were washed thoroughly with ELISA buffer (0.8% phoshate buffered saline plus 0.05% Tween 20) and blocked with 1% (w/v) skimmed milk (Marvel) for 30 min at room temperature. Samples of serum were serially diluted, added to appropriate wells and incubated for 1 h at room temperature. Sera with a predetermined titre for *S. uberis* specific IgG_1, IgG_2 and IgM were used as positive controls whilst pre-colostral serum was used as a negative control. These standards were included in each assay. The following mouse anti-bovine isotype specific monoclonal antibodies were added (1 h, room temperature) where appropriate: IgG_1 (B37) at 1 in 100 000, IgG_2 (B192) at 1 in 30 000 and IgM (B67) at 1 in 40 000. The antibodies were visualised with biotinylated goat anti-mouse IgG (Amersham) at 1 in 2000 (1 h, room temperature) followed by streptavidin-HRP (Amersham) at 1 in 2000 (1 h, room temperature). The substrate used was O-phenylenediamine (3.4 mg ml^{-1}) in citrate buffer (0.05 M citric acid, 0.1 M di-sodium hydrogen orthophosphate and 0.03% (w/v) hydrogen peroxide). The reaction was stopped by the addition of 50 μl 1 M sulphuric acid once the positive standard had developed a strong colour (1–2 min). The changes in absorption were measured at A492 nm in an Anthos ELISA plate reader (Denley Instr. Co. Ltd). Titres were calculated as the highest dilution of sample with an OD value which was 1.5 times the mean OD value of the negative standard.

Lymphocyte profileration in response to S. uberis antigen

Mononuclear cells were isolated from heparinised peripheral blood by density gradient centrifugation (Histopaque 1083, Sigma). The cells collected from the interface were washed three times and resuspended to a final concentration of 1×10^6 ml^{-1} in RPMI 1640 culture medium (Gibco) supplemented with 2 mM L-glutamine, 0.45 sodium bicarbonate, 20 mM HEPES, 100 μg ml^{-1} streptomycin, 100 U ml^{-1} penicillin and 10% (v/v) foetal calf serum (heat-inactivated at 56°C, 1 h). Triplicate cultures were established in 96-well, round-bottomed microtitre trays. Cells (200 μl) were cultured in the presence of 20 μl *S. uberis* antigen (surface extract preparation) diluted in RPMI. Control cultures received an equal volume of RPMI alone or of an optimal dose of the mitogen phytohaemagglutinin (Wellcome Diagnostics) which provided a positive control for the assay. Cultures were maintained at 37°C in 5% CO_2 in air for 5 days. Methyl tritiated thymidine (1 μCi ^{3}H, Tdr, Amersham) was added to each well for the last 6 h of the culture period. Cells were harvested onto glass fibre discs and thymidine incorporation measured by liquid scintillation counting. Results were expressed as the stimulation index (counts per minute (cpm) of cultures containing *S. uberis* antigen divided by cpm of unstimulated controls).

Measurement of opsonic capacity of serum and milk

Serum and milk samples were used in a phagocytosis assay as described by Williams et al. [12]. Briefly, 0.2 ml neutrophils (1×10^7 ml^{-1}) were mixed with 0.1 ml *S. uberis* (6×10^6 ml^{-1}) and 0.3 ml serum (20%) or milk. Control samples contained 0.2 ml phosphate-buffered saline (PBS) instead of neutrophils. The mixtures were rolled (coulter roller) at 37°C for 90 min, diluted in saline, inoculated onto blood agar plates containing Aesculin (0.1% w/v) and incubated overnight at 37°C. The survival of *S. uberis* was determined by counting the number of cfu remaining at the end of the incubation period compared to control samples without neutrophils. Where indicated, the final opsonin concentration of milk or serum was increased to 95% by using stock suspensions of neutrophils at a concentration of 1×10^8 ml^{-1}, and *S. uberis* at 6×10^7 ml^{-1}.

Intramammary bacterial challenge

All animals were challenged 2 to 6 weeks into lactation by infusion of 1 ml of pyrogen-free saline containing approximately 300 cfu *S. uberis* strain 0140J into two mammary quarters (left fore and right hind), animals were challenged after the afternoon milking. For 2 days prior to, and for 7 days after, bacterial challenge, daily milk yields were recorded and quarter milk samples were collected aseptically prior to each milking for bacteriology and cytology. Rectal temperature was recorded together with the appearance of the milk, condition of the udder and the animal's general state of health. Quarter milk samples were not taken for bacteriology and cytology after the start of antibiotic therapy.

Somatic cell counting of milk

Milk samples were fixed with Somafix (Coulter Electronics) and the number of somatic cells present were counted electronically in a Model F_N counter (Coulter Electronics).

Bacteriological examination of milk

Initially, undiluted milk samples (50 μl) were plated onto nutrient agar (Oxoid) supplemented with 5.0% (v/v) washed bovine erythrocytes and 0.1% (w/v) aesculin and incubated for 18 h at 37°C. Once it was clear that an aesculin-hydrolysing Gram-positive cocci had established a new infection, the bacterial numbers were determined by serially diluting milk samples in physiological saline and plating onto the same medium. The Gram-positive cocci isolated from each new infection were stored at 4°C and a single colony from each plate analysed by the DNA fingerprinting technique of Hill and Leigh [13] to ensure that the challenge strain was the cause of the new infection.

Clinical mastitis and therapy

Quarters producing milk with a high bacterial count ($> 1 \times 10^4$ ml^{-1}) during the previous day, and clinical signs throughout the drawing of 25 ml foremilk were given a course of antibiotic therapy (Orbenin QR, Smith Kline Beecham) at the end of that milking. At the end of the experiment (9 days post-challenge) antibiotic was administered to any quarters known to be bacteriologically positive and not previously treated.

Statistics

Student's *t*-tests were performed on the geometric means of the logarithmically transformed data and on the arithmetic means of untransformed data using the Minitab statistical package (Minitab Inc). Means were considered to be significantly different when the probability P was < 0.05.

Results

S. uberis specific antibody responses following vaccination

The levels of specific antibody to *S. uberis* in the serum of all animals prior to vaccination were low, and subsequent alterations following vaccination are shown in Fig. 1. The levels of IgG_1 or IgM in serum following vaccination did not increase. There was, however, a statistically significant ($P < 0.029$) elevation of IgG_2 in the serum of animals which had been given the live vaccine when compared to controls.

Milk samples were pooled from all four quarters at 14 days before drying off and prior to experimental challenge. The levels of *S. uberis*-specific antibody which were detected in the milk samples prior to vaccination were much lower than those measured in serum. No significant elevation in *S. uberis* specific IgG_1, IgG_2 or IgM was seen in samples collected following vaccination and prior to challenge (Fig. 2).

Lymphocyte proliferation

Prior to vaccination, lymphocytes isolated from animals in all 3 groups showed a mean stimulation index of < 7 in response to *S. uberis* antigen in vitro, and the mean responses from all animals in the control group remained below 5 throughout (Fig. 3). Mean responses of cows vaccinated with the surface extract rose slightly following the second administration of antigen but had declined by the time of challenge. In contrast, the mean stimulation index of lymphocytes from cows vaccinated with live *S. uberis* rose progressively following each vaccination and had reached 33.5 prior to challenge. However, due to the considerable variation seen from animal to animal, these differences were not statistically significant. Duplicate assays indicated that the responses were not a result of non-specific mitogenic stimulation by the antigen in vitro (data not shown).

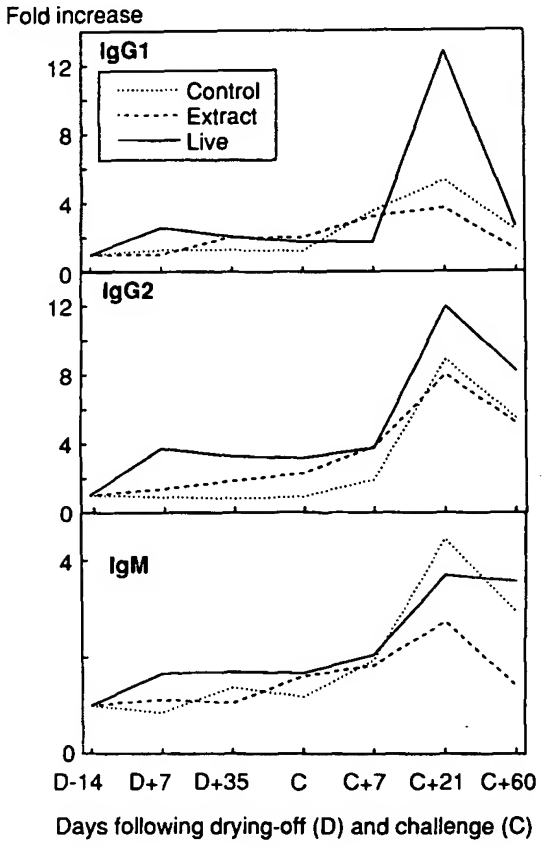

Fig. 1. Alterations in the mean levels of *S. uberis* specific antibody in serum following vaccination during the dry period (D) and subsequent challenge (C) during lactation. Results are expressed as the fold increase in titre compared to pre-vaccination levels. (Mean pre-vaccination titres for all three groups were as follows: IgG_1 439.5, IgG_2 3020 and IgM 233.9).

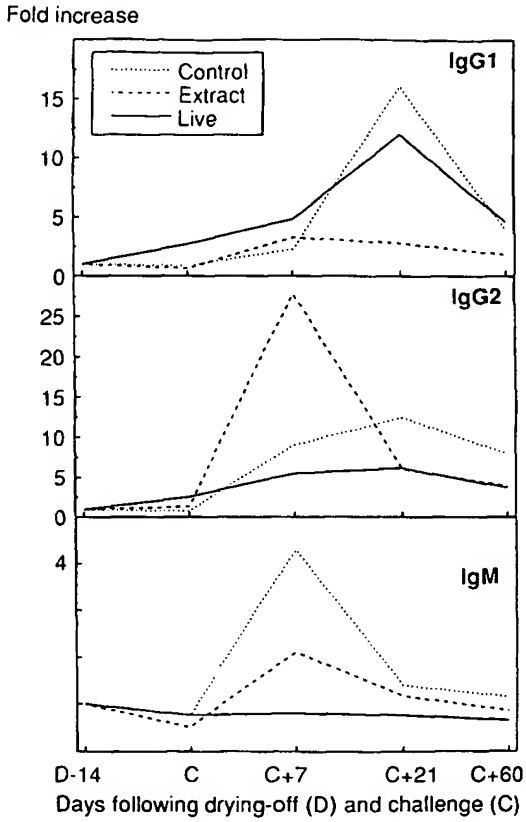

Fig. 2. Alterations in the mean levels of *S. uberis* specific antibody in milk following vaccination during the dry period (D) and subsequent challenge (C) during lactation. Results are expressed as the fold increase in titre compared to pre-vaccination levels. (Mean pre-vaccination titres for all three groups were as follows: IgG_1 6.1, IgG_2 6.0 and IgM 6.0).

Experimental challenge of mammary quarters

All challenged quarters of the animals in the control and extract vaccine groups developed clinical mastitis and required antibiotic therapy between 2–3 and 4–5 days post-challenge, respectively. Both the mean somatic cell count and the number of bacteria present in the milk were lower in the the animals vaccinated with extract than in controls (Fig. 4), but these differences were not significant. The severity of the disease was indistinguishable in these two groups, although the clinical signs (clotted or discoloured milk and swollen udder) developed 24–48 h later in those vaccinated with surface extract. The mean of the loss in milk yield was less in the extract vaccine group than the controls (Fig. 4), but the variation between animals was considerable.

In contrast, the live vaccine regime dramatically modified the pathogenesis of the disease when compared with the control group (Fig. 4). Of the 8 quarters challenged, 3 did not develop clinical mastitis, although 2 of the quarters did show intermittent excretion of bacteria for the first 2 days post-challenge. Three further quarters intermittently excreted low numbers of bacteria (10^2–10^3 ml^{-1} of milk) for the first 4 days but the numbers gradually increased so that by day 9, although no clinical signs could be detected in the milk or gland, these quarters were treated with antibiotics. The remaining two quarters developed clinical mastitis; one on day 4 and the other 15 days post-infection. Direct plating of the milk from the latter gland at 9 days post-challenge indicated that it was free of bacteria; however, DNA finger-printing [13] confirmed that the organism isolated from the gland at the onset of clinical disease was the same as the challenge strain.

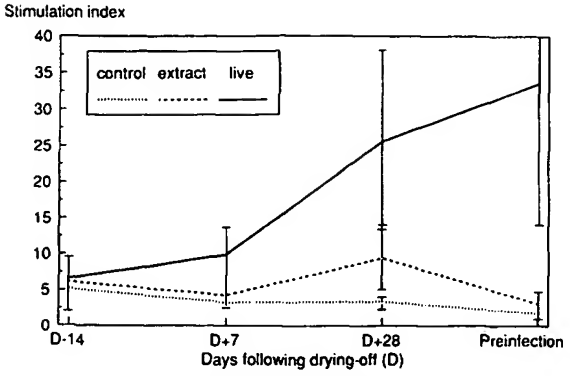

Fig. 3. Lymphocyte responses to *S. uberis* antigen following vaccination during the dry period and immediately prior to infection. Results are expressed as the mean stimulation index for the three groups of cows.

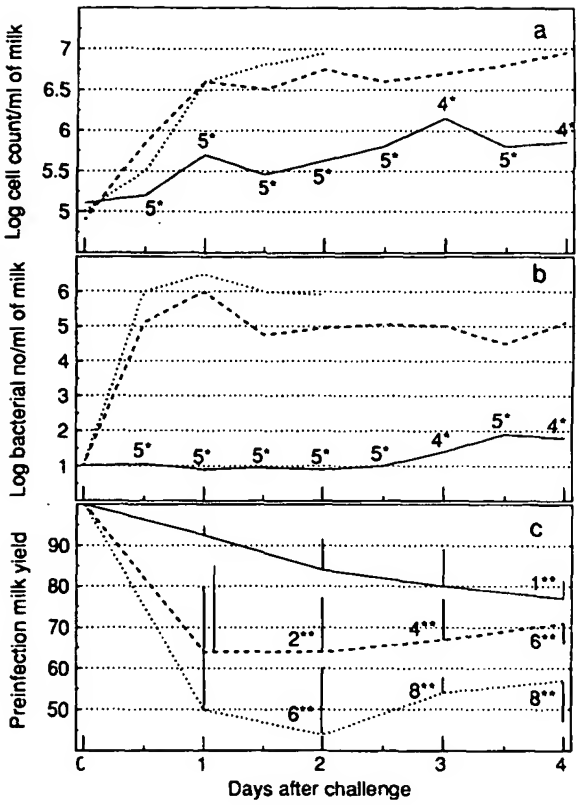

Fig. 4. (a) Mean log somatic cell counts in milk samples collected from each group of cows prior to routine milking. The left fore and right hind mammary quarters of each group (4 animals per group) were given an intramammary challenge of 300 colony forming units of *S. uberis* after the milking at $t = 0$. (b) Mean log numbers of *S. uberis* in milk samples. (c) Mean daily milk yields of cows in each group expressed as the % of the daily yield prior to intramammary bacterial challenge. *: numbers of quarters free of bacteria; **: quarters receiving therapy. There were 8 quarters in results for each treatment.

During the first 2 days post-challenge the mean milk yield of animals in the control and extract vaccine groups showed a sharp fall (Fig. 4), followed by a gradual increase following the start of antibiotic therapy. In contrast, the mean milk yield of the live vaccine group showed only a gradual fall and in one cow whose challenged quarters were both free from infection, no loss in milk production was recorded.

S. uberis specific antibody responses following experimental challenge

The levels of IgG_1 in the serum of control and extract vaccinated cows were unchanged following challenge, whilst cows given the live vaccine showed a marked increase in *S. uberis*-specific IgG_1 three weeks post-infection (Fig. 1). This difference was not, however, statistically significant. *S. uberis*-specific IgG_2 titres were elevated in the serum of cows in all three groups 3 weeks post-challenge and remained high, whilst there was a slight increase in specific IgM in the live and extract-vaccinated animals post-challenge.

Milk samples were collected from challenged and non-challenged quarters and *S. uberis* specific antibody of all three isotypes was seen to increase post-challenge (Fig. 2). IgG_1 levels were raised in milk collected from challenged quarters of cows given the live vaccine and from control animals 3 weeks post-challenge, but not in the milk of extract-vaccinated animals. These levels had declined in samples collected 2 months post-challenge. IgG_1 levels in milk from non-challenged quarters showed the same pattern although the titres were lower than their challenged counterparts (data not shown).

IgG_2 levels were raised in the milk from all challenged quarters, the most pronounced increase being in samples obtained 1 week post-challenge from cows which had been vaccinated with surface extract. Elevated IgG_2 titres were also seen in non-challenged quarters of control animals and to a lesser extent in those of live and extract vaccinates (data not shown).

Elevated levels of IgM were detected in the milk collected from challenged quarters of control animals 1 week post-challenge when compared to those from vaccinated animals ($P = 0.016$), but had declined again in samples collected 3 weeks post-challenge. Levels were raised slightly in the milk from challenged quarters of cows given the extract but not from those given the live vaccine (Fig. 2). IgM levels remained unaltered in non-challenged quarters from all three groups post-infection (data not shown).

Opsonic capacity of serum and milk

Assays to determine the opsonic capability of serum and milk samples failed to demonstrate any changes following vaccination or post-challenge in any of the groups. There were still no discernable differences in any of the groups when the assay was modified to test serum and milk at a final concentration of 95% (data not shown). In all assays *S. uberis* (strain 0140J) showed at least 50% survival following incubation in the presence of bovine PMN.

Discussion

The parenteral immunisation of cattle with live *S. uberis* grown in a casein-supplemented defined medium significantly modified the pathogenesis of the mastitis seen following an experimental intramammary challenge with the same strain. This vaccine regime appeared to modify the speed of replication of the bacteria within the gland, as shown by the difference in bacterial recovery 12 h after intramammary challenge, when the bacterial numbers in the milk of the control and extract vaccine animals was a factor of 10^5 higher than those given the live vaccine. The control of bacterial growth was not associated with the infiltration of neutrophils into the mammary gland which occurred in all animals of the three treatment groups in response to high bacterial numbers. Since this was never associated with a reduction of the number of bacteria in the milk it suggests that neutrophils were ineffective at controlling infection caused by this organism in vivo and contrasts with the known mechanisms of control of other bacteria within the mammary gland, particularly that associated with *E. coli* [6], where killing by neutrophils is essential to control bacterial numbers.

Vaccination with live organisms did not induce a marked increase in *S. uberis* specific antibody in milk of these animals prior to challenge. Serum IgG_2 levels were elevated slightly post-vaccination and increased in all three groups of cows following infection. The levels of IgG_2 in milk varied considerably between the three groups post-challenge and are more likely to reflect the disruption of the blood/mammary gland barrier due to disease in individual animals rather than a specific alteration in immunoglobulins as a consequence of vaccination. Despite the increased levels of IgG_2, no increase in opsonic activity could be detected in serum or milk samples following vaccination or the subsequent challenge, and may explain the inadequacies of mammary neutrophils to control bacterial numbers in vivo. Although IgG_2 was the predominant opsonic isotype for ovine and bovine neutrophils [5,14], IgM was also shown to have opsonic capability against *S. aureus* [15] and *E. coli* [16]. An increase in *S. uberis* specific IgM was also observed following experimental challenge and yet milk and serum samples collected at that time did not show enhanced opsonic activity. This is consistent with a previous report [10], which indicated that *S. uberis* strain 0140J is not readily phagocytosed by bovine neutrophils following growth in the presence of casein or casein derived amino acids and this was considered to be a consequence of the lack of opsonic activity in the milk and/or serum. Lymphocytes isolated from the blood of animals given the live vaccine showed strong proliferative responses to *S. uberis* antigen in vitro following each inoculation. However, due to the small number of animals tested, these responses were not significantly different from control or extract-vaccinated cows. The antigen used did not exert a non-specific, mitogenic effect on the cells, therefore these responses indicate that the systemic administration of live *S. uberis* had succeeded in priming circulating lymphocytes to respond to *S. uberis* antigen in vitro. It is also possible that the local administration of the surface extract preparation seven days into the dry period may have primed the local lymphocyte populations whose response may have contributed to the modified pathogenesis of the disease. The responses of lymphocytes from the mammary gland to *S. uberis* antigen following vaccination requires investigation.

Immunisation of cows with the soluble surface extract of *S. uberis* failed to increase specific serum antibody and no opsonic activity was detected in their serum or milk and lymphocyte responses to *S. uberis* antigen in vitro were not enhanced. However, the extract preparation was capable of in vitro stimulation of lymphocytes from animals which had been primed with live bacteria.

In conclusion, the data described in this paper demonstrates that the pathogenesis of *S. uberis* mastitis in the dairy cow may be dramatically modified by parenteral immunisation with live bacteria. Furthermore, this modification, resulting in lower numbers of bacteria in the milk of challenged cows, was achieved without the apparent involvement of either the neutrophil, specific antibody or any inflammatory response, although the peripheral lymphocytes showed an increased responsiveness to bacterial antigens. This is a novel observation which requires further investigation.

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Further studies on the efficacy of a live vaccine against mastitis caused by *Streptococcus uberis*

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Three groups of dairy cows were immunized by subcutaneous (s.c.) administration of a preparation of live Streptococcus uberis *(strain 0140J) and an intramammary infusion of a soluble surface extract derived from same the bacteria. Animals in Groups 1 and 2 received two s.c. vaccinations plus an intramammary inoculation. Animals in Group 3 received two s.c. vaccinations but did not receive the intramammary infusion. In addition to the vaccinated animals, each group also contained two non-vaccinated (control) animals. All animals were challenged experimentally by intramammary infusion (in two quarters per animal) of* ca *100 c.f.u. of* S. uberis *(strain 0140J or C221) and monitored for clinical signs of disease, bacterial numbers in milk, somatic cell count in milk, and daily milk yield for the following 10 days. Animals in Group 1 were challenged with strain 0140J. Only one out of six challenged quarters of three vaccinated cows developed clinical disease compared to all (four out of four) quarters of non-vaccinated cows. Animals in Group 2 were challenged with strain C221. All challenged quarters of three vaccinated (six out of six) and two non-vaccinated (four out of four) cows developed clinical mastitis. Animals in Group 3 were challenged with strain 0140J. Five out of eight quarters on four vaccinated cows developed clinical mastitis but the onset was delayed in comparison with that in both non-vaccinated cows in which four out of four challenged quarters developed clinical mastitis. These results indicated that vaccination with live* S. uberis *protects against challenge with the homologous strain but was less effective against a heterologous strain. Reduced protection was also seen when the intramammary booster was omitted.*

Keywords: bovine; mastitis; *Streptococcus uberis*

Streptococcus uberis is a common cause of bovine mastitis and is responsible for *ca* 20% of all clinical cases in the UK[1]. Although control recommendations have effectively reduced the incidence of mastitis due to contagious pathogens such as *Staphylococcus aureus*, *Streptococcus agalactiae* and *Streptococcus dygalactiae*, they have had little impact on the incidence of mastitis caused by environmental pathogens such as *S. uberis*[2,3]. Strategies based upon vaccination to prevent infection have therefore become highly desirable. Initial studies indicated that cows which had experienced a previous intramammary infection with *S. uberis* demonstrated a reduction in clinical mastitis following subsequent experimental challenge[4]. The mechanism by which this effect was attained was not known, although results obtained in other laboratories had indicated that enhanced levels of opsonic antibody and the rapid recruitment of neutrophils into the mammary gland are required for protection of the mammary gland against infection by *S. aureus* and *Escherichia coli*[5–7]. Subsequent studies in which cattle were immunized with a subcutaneous (s.c.) preparation of live *S. uberis* (strain 0140J) plus an intramammary booster with bacterial surface extract derived from *S. uberis*, showed that protection could be achieved against experimental challenge with the same strain[8]. Protection against infection was achieved in the absence of opsonic antibody and without a marked influx of neutrophils into the mammary gland following challenge. The experiments reported in this communication were intended to determine the potential contribution of the intramammary booster to protection and to determine the extent of the protection against challenge with a non-vaccine strain of *S. uberis* (strain C221).

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MATERIALS AND METHODS

Animals

Sixteen Friesian dairy cows from the Institute's dairy herd and with no history of mastitis caused by *S. uberis*

were divided into three groups on the basis of their expected calving date. Groups 1 and 2 comprised three animals which received appropriate vaccinations and two non-vaccinated, controls, whilst Group 3 comprised four vaccinates and two non-vaccinated controls.

Bacteria

S. uberis strains 0140J and C221 (originally isolated from cases of bovine mastitis) were used as indicated. Both organisms were stored in Todd Hewitt broth (Oxoid) at −20°C in the presence of 25% (v/v) glycerol. For inoculation of chemically-defined medium or infection of cattle, bacteria were grown in Todd Hewitt broth at 37°C for 18 h.

Vaccine preparations

Live *S. uberis* strain 0140J and bacterial surface extract were prepared as described by Hill *et al.*[8]. Briefly, bacteria were inoculated into chemically-defined medium[9], grown overnight at 37°C, harvested during late exponential growth and washed twice in phosphate buffered saline (PBS). Live bacteria were then resuspended in 10% of the original culture volume to give a final concentration of 10^{10} c.f.u. ml^{-1} and stored at −70°C until required. Bacterial surface extract was prepared from the bacterial suspension using mutanolysin and type X hyaluronidase as described by Hill *et al.*[8]. Resulting protoplasts were removed by centrifugation (7000*g*, 20 min) and the supernatant (extract) collected, sterilized by filtration and stored at −70°C until required.

Vaccination regime

Animals were vaccinated according to the published protocol[8]. Vaccinates in Groups 1 and 2 received two s.c. vaccinations [each comprising 10^{10} c.f.u. *S. uberis* strain 0140J plus 1 ml Freund's incomplete adjuvant and 2 ml 0.1% Tween 20, one 14 days before drying off and the second within 48 h of calving. These animals were also given an intramammary inoculation comprising 5 ml of the cell wall extract prepared from the same bacterial cells, into all four quarters 7 days into the dry period. This intramammary inoculation did not contain adjuvant material. Animals in Group 3 received the two s.c. vaccinations of live *S. uberis* (as above) but did not receive the intramammary infusion of cell wall extract during the dry period.

Experimental challenge

All animals were challenged with the appropriate strain of *S. uberis*, 2–6 weeks into lactation. Each animal received a 1 ml inoculum containing *ca* 100 c.f.u. in PBSa into each of two mammary quarters, whilst a third quarter was inoculated with 1 ml PBSa only. Sterile quarter milk samples were collected at each milking until the end of the 10 day experiment. The numbers of bacteria and somatic cells present in the milk were determined and the milk yields recorded. Any quarters displaying clinical signs (clotted/discoloured foremilk, swollen and tender quarter) and known to be bacteriologically positive were given a course of antibiotic therapy (Leo Yellow). Any infected quarters which had not already been treated were given a course of antibiotics at the end of the experiment.

Determination of *S. uberis* specific antibody in serum and milk

Levels of specific antibody in serum and milk were determined by ELISA[8]. Briefly, microtitre plates were coated with the bacterial suspension (above) diluted in 0.05 M carbonate buffer, blocked with 1% (w/v) skimmed milk, and serum and milk samples added to appropriate wells. Mouse anti-bovine isotype specific monoclonal antibodies were then added and visualized by the addition of biotinylated goat anti-mouse IgG followed by streptavidin-HRP. Plates were developed using 3,3',5,5' tetramethylbenzidine (TMB; Kirkegaard and Perry Labs Inc.), the reaction was stopped by the addition of 1 M sulphuric acid and the changes in optical density measured at 492 nm using an ELISA plate reader. Samples of serum and milk were obtained prior to vaccination and post-vaccination (immediately prior to challenge).

RESULTS

Group 1. Subcutaneous and intramammary vaccination with *S. uberis* strain 0140J followed by challenge with the same strain

Vaccinates in Group 1 had been immunized with live *S. uberis* strain 0104J plus the intramammary booster and were challenged with the homologous strain. Bacteria were recovered in large numbers from the milk of all four quarters on two non-vaccinated animals (*Figure 1*a). Clinical signs were evident in three of these quarters within 48 h of challenge and were treated with antibiotic at the next milking. The fourth quarter did not display any clinical signs until 7 days post-challenge and was treated with antibiotic at milking 15. In contrast, *S. uberis* was never recovered from three of the six challenged quarters on vaccinated animals and the mean numbers of bacteria recovered were very low (*Figure 1*a). Two quarters on a single animal shed low numbers of bacteria for a short time before clearing the infection in the absence of any clinical signs. Bacteria were also recovered from a single quarter of another vaccinated animal although the number recovered was reduced during the first seven milkings post-challenge when compared to those from non-vaccinated animals. Clinical signs were not evident in this quarter until milking 11 and at this time the quarter was treated with antibiotics.

Mean somatic cell counts increased markedly in control animals within 48 h of challenge (*Figure 1*b), whilst only a slight rise in mean cell count was seen in the milk of vaccinated cows. Somatic cell counts were elevated in all quarters from which bacteria were recovered, although in the case of vaccinated animals, these rises were much less than that seen in controls, the increase was transient and followed the recovery of bacteria from the milk (*Figure 1*b). The dramatic fall in the milk yield of control animals (*Figure 1*c) coincided with the onset of clinical signs whilst the mean milk yield in vaccinated animals did not fall below 85% of that prior to challenge.

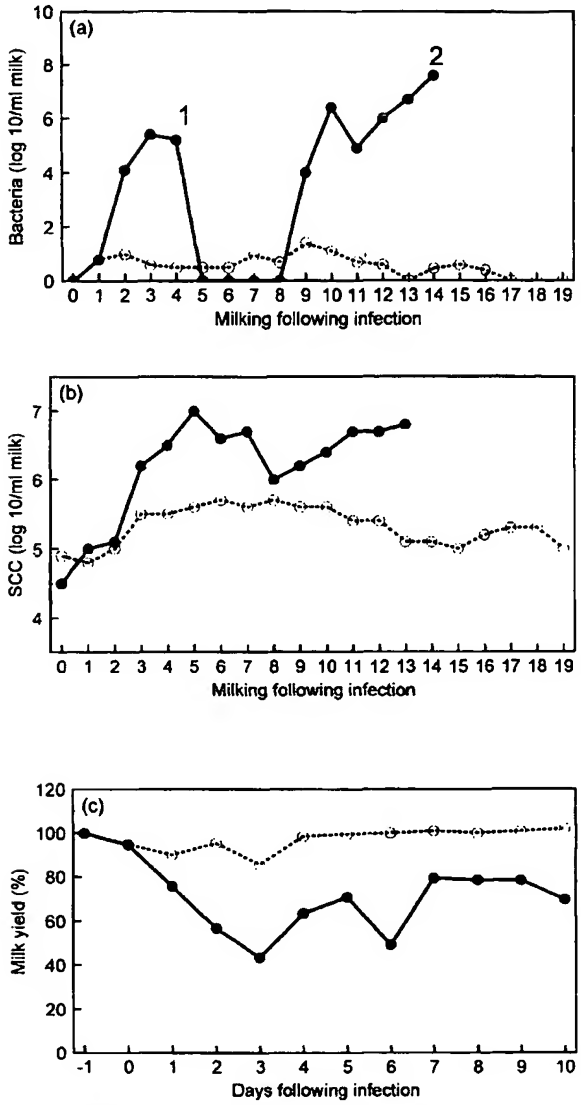

Figure 1 Mean bacterial recovery (a), somatic cell count SCC (b) and alterations in milk yield (c) of control (———) and vaccinated (- - -) cows in Group 1. Vaccinates were immunized with *S. uberis* strain 0140J and all cows were challenged with strain 0140J during lactation. *n*=Four quarters on two control cows, *n*=six quarters on three vaccinated cows. [1]Three out of four quarters treated, [2]remaining quarter treated

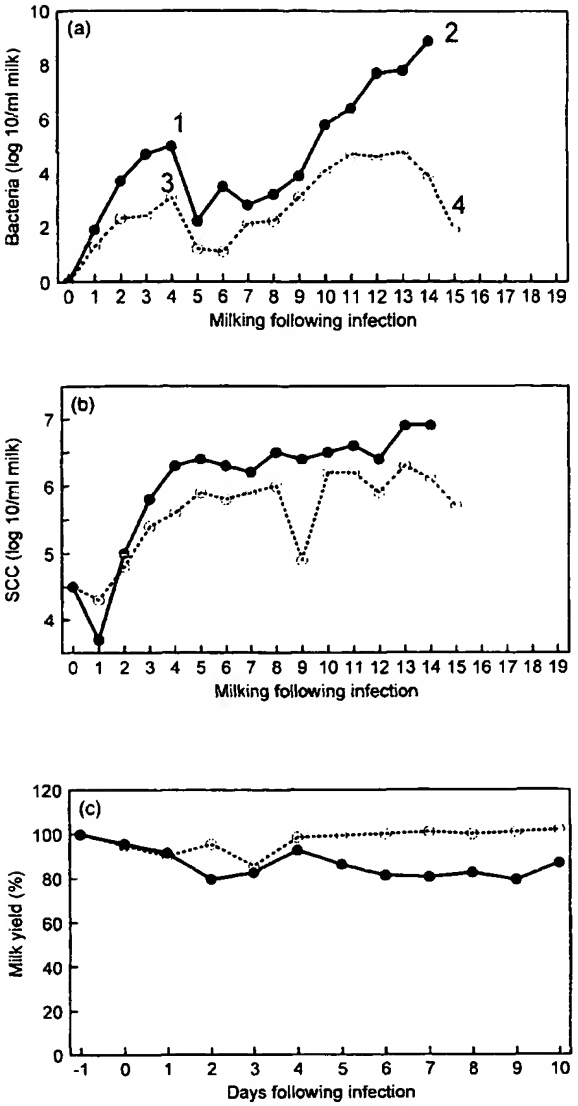

Figure 2 Mean bacterial recovery (a), somatic cell count SCC (b) and alterations in milk yield (c) of control (———) and vaccinated (- - -) cows in Group 2. Vaccinates were immunized with *S. uberis* strain 0140J and all cows were challenged with strain C221 during lactation. *n*=Four quarters on two control cows, *n*=six quarters on three vaccinated cows. [1]One out of four quarters treated, [2]three remaining quarters treated, [3]one out of six quarters treated, [4]five remaining quarters treated

Group 2. Subcutaneous and intramammary vaccination with *S. uberis* strain 0140J and challenge with strain C221

Vaccinates in Group 2 had been immunized with live *S. uberis* strain 0104J plus the intramammary booster but were challenged with *ca* 100 c.f.u. *S. uberis* strain C221. Bacteria were recovered in large numbers from the milk of all four quarters on two non-vaccinated cows (*Figure 2*a), one quarter required antibiotic therapy at milking 4, although clinical signs in the three remaining quarters only became evident around the 12th milking post-challenge. By comparison, the mean recovery of *S. uberis* was lower in the challenged quarters on vaccinated cows for the first three to six milkings post-infection (*Figure 2*a). By milking 5, only one out of six challenged quarters had developed clinical signs of mastitis and this quarter required treatment at this time. Subsequently, the mean bacterial numbers gradually increased until they became sufficient to induce clinical signs and the five remaining quarters were treated at milking 15.

Somatic cell counts were elevated in all quarters from which bacteria were recovered in both vaccinated and non-vaccinated animals and there was little difference in the mean cell counts of the two groups (*Figure 2*b). The mean milk yield fell by 20% in non-vaccinated animals challenged with strain C221 (*Figure 2*c), compared with a fall of around 60% in similar animals challenged with strain 0140J. An additional fall in milk yield was observed in one cow 6–7 days post-challenge; this coincided with a loss of condition and appeared not to be

directly related to the experimental infection. The mean milk yield of vaccinated cows fell to 80% of that recorded prior to challenge (*Figure 2*c) and this fall was mainly a result of the clinical infection in the single quarter of one vaccinated cow.

Group 3. Subcutaneous vaccination and challenge with *S. uberis* strain 0140J

The vaccinates in Group 3 had been immunized with live *S. uberis* strain 0104J but did not receive the intramammary infusion of cell surface extract 7 days into the dry period. These cows were subsequently challenged with *ca* 100 c.f.u. *S. uberis* strain 0140J in order to determine the importance of the intramammary vaccination in inducing the level of protection attained in Group 1.

Bacteria were recovered in large numbers from the milk of all four quarters of non-vaccinated cows (*Figure 3*a) and clinical signs became evident within 24 h post-challenge. All quarters from these animals had received antibiotic therapy by the end of milking 6. By comparison, there was a marked reduction in the mean numbers of bacteria recovered from the milk of vaccinated cows (*Figure 3*a). Three of the eight challenged quarters on vaccinated cows shed only low numbers of bacteria and two of these quarters cleared the infection without displaying any clinical signs or need for antibiotic therapy. The number of bacteria recovered from the five remaining quarters did, however, increase following challenge such that all quarters developed clinical signs between milking 4 and milking 10 and these quarters required antibiotic therapy.

As in Groups 1 and 2, somatic cell counts were elevated in all quarters from which bacteria were recovered and there was little difference in the magnitude of the mean counts of the control and vaccinated cows (*Figure 3*b). The milk yield of control animals was depressed by 40% 2–3 days post-challenge but recovered following antibiotic therapy (*Figure 3*c), whilst that of vaccinated cows fell by only 10–20% following challenge.

Levels of *S. uberis* specific antibody in serum and milk

Mean levels of *S. uberis* specific IgG1 and IgG2 were increased in the serum of vaccinated animals to between 2- and 20-fold of that seen prior to vaccination. Those in non-vaccinated animals remained low. None increased more than threefold during the period prior to challenge (*Table 1*). In contrast, there was relatively little change in the mean levels of specific IgG1 or IgG2 in the milk of vaccinated cows (*Table 2*) and with the exception of the levels of IgG2 in Group 2 and the levels IgG1 in Group 3 there was no discernable elevation in the local antibody responses of vaccinated animals. The levels of IgG1 and IgG2 in milk from non-vaccinated animal remained similar throughout this period (*Table 2*).

DISCUSSION

Results obtained from the animals in Group 1 confirmed the effect of the published vaccination protocol comprising parenteral vaccination of dairy cows with live *S. uberis* plus an intramammary infusion of bacterial surface extract[8], and showed that this was capable of protecting the mammary gland from subsequent experimental challenge with the same strain. These results consolidate and, in fact, supersede those obtained in the previous study since the difference between controls and vaccinates for all the parameters measured (bacterial recovery, somatic cell counts, alterations in milk yield and clinical signs) was much greater. This suggests that s.c. immunization with a live, or possibly an attenuated live, vaccine may be a viable proposition for the prevention of mastitis caused by this organism.

The clinical and bacteriological data obtained from Group 2 following the challenge of vaccinated animals with heterologous strain C221 suggests that the initial colonization of the mammary gland by *S. uberis* strain C221 was delayed in vaccinated animals when compared

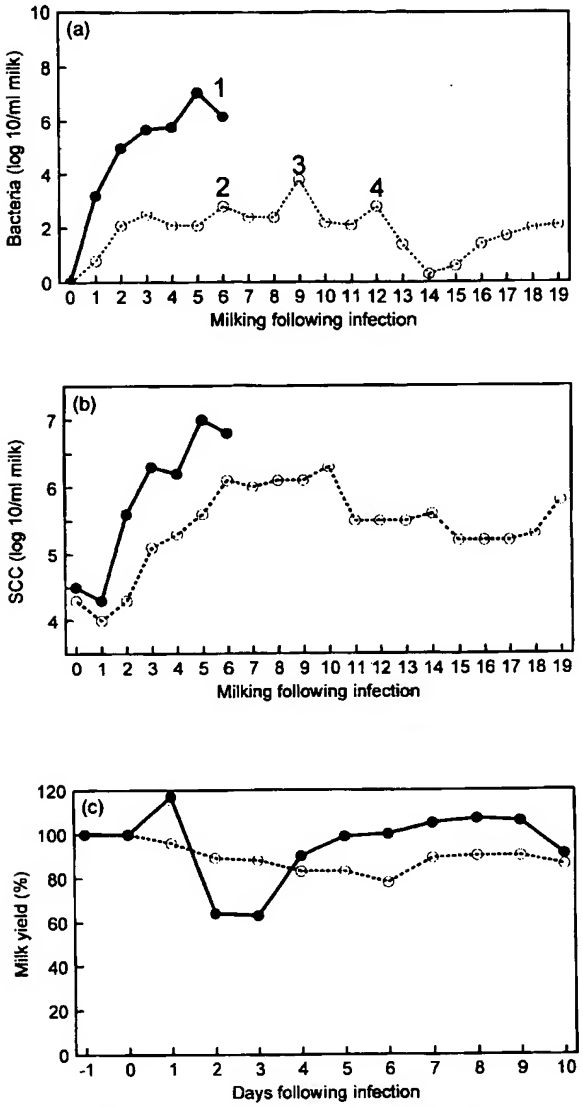

Figure 3 Mean bacterial recovery (a), somatic cell count SCC (b) and alterations in milk yield (c) of control (———) and vaccinated (- - -) cows in Group 3. Vaccinates were immunized with *S. uberis* strain 0140J but did not receive the intramammary booster, and all cows were challenged with strain 0140J during lactation. *n*=Four quarters on two control cows, *n*=eight quarters on four vaccinated cows. [1]All four quarters treated, [2]one out of eight quarters treated, [3]two out of seven remaining quarters treated, [4]two out of five remaining quarters treated

Table 1 Mean levels of *S. uberis* specific IgG1 and IgG2 in serum

	S. uberis specific antibody in serum		
	Pre-vaccination	Pre-challenge	Fold increase
IgG1			
Group 1			
Controls	4926	7435	1.51
Vaccinates	10202	31414	3.08
Group 2			
Controls	5884	13839	2.35
Vaccinates	6180	122313	19.79
Group 3			
Controls	11243	13504	1.20
Vaccinates	8575	172195	20.08
IgG2			
Group 1			
Controls	11368	13923	1.22
Vaccinates	14115	59763	4.23
Group 2			
Controls	41717	32165	0.77
Vaccinates	30490	81863	2.68
Group 3			
Controls	45210	63700	1.41
Vaccinates	27195	226813	8.34

Table 2 Mean levels of *S. uberis* specific IgG1 and IgG2 in milk (values represent the mean of samples collected from all four quarters of each animal)

	S. uberis specific antibody in milk		
	Pre-vaccination	Pre-challenge	Fold increase
IgG1			
Group 1			
Controls	134	97	0.72
Vaccinates	1688	694	0.41
Group 2			
Controls	1165	412	0.35
Vaccinates	257	351	1.36
Group 3			
Controls	297	193	0.64
Vaccinates	513	2662	5.19
IgG2			
Group 1			
Controls	24	45	1.87
Vaccinates	141	302	2.14
Group 2			
Controls	113	171	1.51
Vaccinates	97	6483	66.80
Group 3			
Controls	183	103	0.56
Vaccinates	111	199	1.79

with controls and that the mean numbers of bacteria recovered remained low ($<10^3$ c.f.u. ml^{-1} milk) for up to seven milkings post-challenge before they finally attained sufficient numbers to cause clinical disease. It was also noted that clinical signs in both control animals and one of the three vaccinates were more extreme than those in the two remaining vaccinates and this appeared to be related to the very high numbers of bacteria recovered from these quarters. These results indicate that vaccination with *S. uberis* strain 0140J may retard bacterial colonization following challenge with a heterologous strain, but that this is insufficient to prevent infection and thereby reduce the incidence of clinical disease. An effective live vaccine may therefore require a cocktail of several different strains of *S. uberis* in order to achieve protection against *S. uberis* mastitis in the field. It should be borne in mind, however, that this is based upon data from challenge with only a single heterologous strain and that protection against other less virulent strains may have been achieved.

The results obtained from Group 3, in which the intramammary infusion of bacterial surface extract during the dry period was omitted, revealed that mean bacterial numbers and the extent of clinical signs were reduced following only two s.c. vaccinations with live *S. uberis*. However, the level of protection achieved (three out of eight quarters) was not as effective as had been attained in Group 1 (five out of six quarters). Whilst there was only a slight reduction in the milk yield of vaccinated animals in Group 3, mean number of somatic cells was raised beyond 10^6 cells ml^{-1} milk, this level is unacceptable to the UK dairy industry[10], and further supports the microbiological and clinical data which suggest that the local administration of cell wall extract does make a significant contribution to the protective effects obtained.

Previous attempts to vaccinate ruminants against mastitis-causing pathogens have been targeted towards the elevation of specific antibody in the milk and the rapid mobilization of neutrophils into the mammary gland in response to infection[5–7]. The timing and route of administration of the live *S. uberis* and the cell wall extract was therefore based upon the protocols used to generate high levels of rotavirus-specific antibody in colostrum[11]. As in the previous study using this vaccine regime[8], *S. uberis*-specific IgG1 was elevated in the serum of vaccinated cows in all three groups and there was little difference in the levels of *S. uberis*-specific antibody in milk, despite the omission of the intramammary booster in Group 3. This suggests that the key virulence determinants for *S. uberis* may not be somatic antigens presented on the bacterial capsule or cell wall, but could be factors produced or secreted by the live bacteria during growth *in vivo*. The production of such factors by *S. uberis* and their potential as vaccine antigens is the subject of current investigations.

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